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(71) Applicant: SYNTEX (USA) INC. [US/US]; Main Stem A2-200, 3401 Hillview Avenue, Palo Alto, CA 94304 (US).			
(72) Inventors: LOU, Lillian, Lien-Li; 955 Harriet Street, Palo Alto, CA 94301 (US). BARNETT, Jimmy, Wayne; P.O. Box D-11, 220 Autumn Street, La Honda, CA 94020 (US).			
(74) Agents: VON MORZE, Herwig et al.; Syntex (USA) Inc., A2-200, 3401 Hillview Avenue, Palo Alto, CA 94304 (US).			
(54) Title: CLONING AND EXPRESSION OF HUMAN GMP SYNTHETASE, ITS USE IN SCREENING FOR INHIBITORS OF HUMAN GMP SYNTHETASE AND INHIBITORS OF HUMAN GMP SYNTHETASE			
(57) Abstract			
<p>One aspect of the present invention is a purified human GMP synthetase and a method of purifying it from naturally occurring sources. Another aspect of the present invention is a recombinant human GMP synthetase as well as DNA sequences coding for human GMP synthetase, expression vectors comprising such a coding sequence, and host cells transformed with these expression vectors capable of producing human GMP synthetase. Also forming part of this invention is a recombinant process for the production of GMP synthetase. Further provided is a method of purifying GMP synthetase from natural or recombinant sources. Other aspects of the invention include antibodies to human GMP synthetase and the use of such antibodies to assay for human GMP synthetase. Another aspect of this invention is the use of purified naturally occurring human GMP synthetase or recombinant human GMP synthetase to identify inhibitors of GMP synthetase activity. Another aspect of the invention is inhibitors of human GMP synthetase activity obtained using purified human GMP synthetase. Such inhibitors preferably have an IC₅₀ of 5 μM or less, more preferably 1 μM or less.</p>			

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Cloning and Expression of Human GMP Synthetase, its use in Screening for Inhibitors of Human GMP Synthetase and Inhibitors of Human GMP Synthetase

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates to purified human GMP synthetase, its production by recombinant technology and nucleic acid sequences encoding for human GMP synthetase and its use in assaying for inhibitors of human GMP synthetase activity and the inhibitors identifiable by such assays.

Human guanosine 5'-monophosphate (GMP) synthetase is a key enzyme in the de novo synthesis of guanine nucleotides. Biosynthesis of guanine nucleotides is not only essential for deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) synthesis, but also for providing guanosine 5'-triphosphate (GTP) which is involved in a number of cellular processes important for cell division. GTP hydrolysis is required for microtubule assembly, protein glycosylation, synthesis of adenine nucleotides, protein translation and activation of G proteins. A key step in the de novo synthesis of guanine nucleotides is the conversion of inosine 5'-monophosphate (IMP) to guanosine 5'-monophosphate (GMP). Two enzymes are involved in converting IMP to GMP: IMP dehydrogenase (EC 1.1.1.205) which catalyzes the oxidation of IMP to XMP (xanthine 5'-monophosphate) and GMP synthetase (EC 6.3.5.2.) which catalyzes the amination of XMP to GTP. The reaction catalyzed by GMP synthetase is:



Both GMP synthetase and IMP dehydrogenase exhibit elevated levels of activity in rapidly proliferating cells, such as neoplastic and regenerating tissues. Inhibition of GMP synthetase or IMP dehydrogenase has been shown to result in the inhibition of cell growth. Because of this anti-proliferative effect of GMP synthetase and IMP dehydrogenase inhibitors, both enzymes are potential targets for anti-cancer and immunosuppressive therapies. In fact, in recent clinical trials, a potent inhibitor of IMP dehydrogenase, mycophenolic acid, was shown to be effective in treatment of transplant rejection and rheumatoid arthritis.

The inhibition of lymphocyte proliferation by mycophenolic acid is closely correlated with the lowering of the intracellular pool of guanine nucleotides. Furthermore, this inhibition of proliferation can be blocked by the addition of exogenous guanosine. These data suggest that the immunosuppressive activity of mycophenolic acid is a result of the depletion of the guanine nucleotide pool. Since GMP synthetase is crucial for the de novo synthesis of guanine nucleotides, the inhibition of GMP synthetase should also result in the depletion of guanine nucleotides and therefore could be a target for immunosuppressive and cancer therapies.

Despite its significance, the information available for human GMP synthetase is limited. Heretofore, the human enzyme has not been obtained in a purified state. Even though a partially purified preparation from human fibroblast cells (T. Page, B. Bakay and W.L. Nyhan; *Int. J. Biochem.*, 16, 117-120, (1984)) has been reported, it contained varying amounts 5'-nucleotidase, purine nucleoside phosphorylase, and GMP kinase. The degree

of purification of the enzyme was approximately 50-fold over the naturally occurring material and its specific activity was reported to be 5990 pmoles guanine compound produced/min/mg. By contrast, the present invention discloses a purified human GMP synthetase purified 936-fold over the naturally occurring material with a specific activity of at least 2,500,000 pmoles adenosine 5'-monophosphate (AMP) produced/min/mg. GMP synthetases of mammalian origin have been isolated in varying degrees of purity from a variety of sources. However, mammalian GMP synthetases, even if available in a high degree of purity, are of limited utility in screening for inhibitors for the purposes of human therapy.

Genetic information for human GMP synthetase is not available. Although the cDNA for GMP synthetase has been isolated from *E. coli*, *B. subtilis* and *D. discoideum*, no human or mammalian cDNAs or genes for GMP synthetase are available. Therefore it has not been possible to produce human GMP synthetase by recombinant DNA technology.

Accordingly, human GMP synthetase has not been available in a form suitable either for elucidation of its mechanism or for its use in screening for potential inhibitors. Neither has there been sequence information such as, for example, cDNA sequences coding for human GMP synthetase which would enable the production of human GMP synthetase by recombinant DNA technology. For these reasons, it is desirable to produce pure, well characterized human GMP synthetase in large quantities for the purposes, for example, of elucidating the mechanisms of its activity, identifying its role in various disease states and screening for therapeutically significant inhibitors of guanine nucleotide biosynthesis. Furthermore, it would be desirable to produce human GMP synthetase by means of recombinant DNA technology because it would make the enzyme available in much larger amounts than is practical in purification from natural sources.

2. Summary of Related Art

Human GMP Synthetase; T. Page, B. Bakay and W.L. Nyhan; Int. J. Biochem., 16, 117-120, (1984); disclosed GMP synthetase from human fibroblasts, purified approximately 50-fold by ammonium sulfate fractionation and gel filtration.

Purification and Characterization of GMP Synthetase from Yoshida Sarcoma Ascites Cells; K. Harai, Y. Matsuda and H. Nakagawa; J. Biochem., 102, 893-902, (1987); disclosed GMP synthetase purified from Yoshida Sarcoma mouse ascites cells by means of procedures including centrifugal fractionation. The purified enzyme was shown to be homogeneous on SDS-polyacrylamide gel electrophoresis and isoelectric focusing in polyacrylamide gel.

GMP Synthetase from Ehrlich Ascites Cells; T. Spector; Methods Enzymology, 51, 219-224, (1978); described coupled spectrophotometric and radiochemical assays for measuring GMP synthetase. The formation of AMP and/or GMP is coupled to the oxidation of NADH as mediated via AMP kinase

and/or GMP kinase, pyruvate kinase, and lactate dehydrogenase.

Nucleotide Sequence of the *guaA* Gene Encoding GMP Synthetase of *Escherichia coli* K12; A. A. Tiedeman, J.M. Smith and H. Zalkin; J. Biol. Chem. 260, 8676-8679, (1985); reported the cloning and DNA sequence of a 1.7-kilobase pair *BanII*-*PvuII* fragment containing the *E. coli guaA* structural gene. A single open reading frame of 1575 nucleotides was found and sequenced. The deduced amino acid sequence contained 525 residues having a calculated subunit M_r of 58,604.

Functional Cloning of a *Dictyostelium discoideum* cDNA Encoding GMP Synthetase; M.M. Van Lookeren Campagne, J. Franke and R.H. Kessin; J. Biol. Chem. 266, 16448-16452 (1991); disclosed a functional cloning procedure to recover a cDNA coding for the GMP synthetase of *Dictyostelium discoideum*. The enzyme is encoded by a single gene. The open reading frame encodes a protein of 718 amino acids with a predicted molecular mass of 79.6 kDa.

Cloning and Sequence of *Bacillus subtilis purA* and *guaA*, Involved in the Conversion of IMP to AMP and GMP; P. Mantsala and H. Zalkin; J. Bacteriology 174, 1883-1890 (1992); disclosed the nucleotide sequences of the *Bacillus subtilis* genes *purA*, encoding adenylosuccinate synthetase, and *guaA*, coding for GMP synthetase. The sequences were determined from a series of gene fragments isolated by polymerase chain reaction amplification, library screening and plasmid rescue techniques.

Guanosine Monophosphate Synthetase from Ehrlich ascites cells: Multiple inhibition by pyrophosphate and nucleosides; T. Spector, T.E. Jones, T.A. Krenitsky and R.J. Harvey; Biochimica et Biophysica Acta 452, 597-607 (1976); disclosed nucleoside inhibitors of GMP synthetase from Ehrlich ascites cells as well as the inhibition of the enzyme by its reaction product, pyrophosphate.

SUMMARY OF THE INVENTION

One aspect of the present invention is a purified human GMP synthetase and a method of purifying it from naturally occurring sources. The method comprises homogenizing host cells producing human GMP synthetase, isolating the cytosolic component, fractionating the cytosolic component to its protein fraction and subjecting the protein component to chromatographic purification. Another aspect of the present invention is a recombinant human GMP synthetase as well as DNA sequences coding for human GMP synthetase, expression vectors comprising such a coding sequence, and host cells transformed with these expression vectors capable of producing human GMP synthetase. In one aspect, the invention includes a nucleic acid molecule included in, or derived from, the sequence: (GMP5.6 (SEQ ID NO: 1)), which is capable of encoding for human GMP synthetase.

Another aspect of the invention is sets of degenerate polynucleotide primers corresponding to spaced amino acid sequences of human GMP synthetase for use in selectively amplifying human derived nucleic acid sequences which code for GMP synthetase. Each set of primers contains

substantially all of the possible coding sequences corresponding to the spaced amino acid sequences of human GMP synthetase. That is, each set includes at least one primer species that can effectively hybridize with the coding sequence of the corresponding amino acid region.

Further aspects of this invention include polynucleotide probes capable of hybridizing to nucleic acid sequences contained within the nucleic acid sequence of human GMP synthetase as well as such polynucleotide probes labeled with at least one reporter molecule. Also included within this invention are assays for DNA sequences encoding for human GMP synthetase using such labeled or unlabeled polynucleotide probes.

Also forming part of this invention is a recombinant process for the production of GMP synthetase. This recombinant process involves inserting a DNA sequence encoding for GMP synthetase into an expression vector, transforming a suitable host with the vector and isolating the recombinant protein expressed by the vector.

Further provided is a method of purifying GMP synthetase from natural or recombinant sources. This method comprises the steps of homogenizing cells expressing human GMP synthetase and isolating the cytosolic component, fractionating the cytosolic component to its protein fraction and subjecting the protein fraction to chromatographic purification.

Other aspects of the invention include antibodies to human GMP synthetase and the use of such antibodies to assay for human GMP synthetase.

Another aspect of this invention is the use of purified naturally occurring human GMP synthetase or recombinant human GMP synthetase to identify inhibitors of GMP synthetase activity. In this aspect an assay is carried out by a method comprising contacting a medium suspected of containing one or more of such inhibitors with purified human GMP synthetase and measuring the activity of the human GMP synthetase.

Another aspect of the invention is inhibitors of human GMP synthetase activity obtained using purified human GMP synthetase. Such inhibitors preferably have an IC_{50} of 5 μ M or less, more preferably 1 μ M or less, and find use in inhibiting human GMP synthetase activity.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Nucleotide and predicted amino acid sequences of human GMP synthetase.

Amino acid residues are represented in single-letter code. Lines and numbers in parentheses mark the position of sequences corresponding to the tryptic peptides determined from the purified native enzyme. Boxes mark the position of sequences corresponding to the oligonucleotide primers. Nucleotide residue 1 represents the start site of translation.

Figure 2. Functional expression of *GMPS.6* (SEQ ID NO: 1) in a GMP synthetase deficient strain of *E. coli*.

The expression vectors NF.6 and ES.6 were transfected into AT2465 *E. coli*,

-5-

and the cultures were spread on minimal media plates with or without guanosine as described in Example 2. Colony formation was monitored after 24 hours. To determine GMP synthetase activity, single colonies were selected, propagated and induced with IPTG, as described in Example 2.

Single colonies were selected from "minus guanosine" plates with NF.6 transfected cells and from "plus guanosine" plates with ES.6 transfected cells. Single colonies of cells that were not transfected were selected from LB plates. Cultures were harvested and the cytosolic fractions were assayed for GMP synthetase activity as described in Example 2.

Figure 3. Purification of GMP synthetase from A3.01 cells.

Samples from each step of the purification were subjected to SDS polyacrylamide gel electrophoresis and stained with Coomassie blue. The purification step and amount of protein loaded in each lane are as follows: lanes 1. 35-60 % ammonium sulfate precipitate, 6 μ g; 2. DEAE-cellulose pool, 2.5 μ g; 3. Phenyl-5PW pool, 3 μ g; 4. Mono Q pool, 3 μ g.

Figure 4. Restriction map of GMPS.6 (SEQ ID NO: 1) and probes.

The region of hatched bars indicates the position of the longest open reading frame. The scale gives length in base pairs. The black bar marks the length and position of the PCR fragment pcr.2S8A. The open bars mark the length and position of the cDNA fragments used as probes in northern and Southern hybridization analysis. The small arrows mark the position and direction of oligonucleotide primers 2S and 8A in the 5' to 3' orientation.

DETAILED DESCRIPTION OF THE INVENTION

The present invention includes purified naturally occurring and recombinant human GMP synthetase as well as methods of purifying human GMP synthetase from cells expressing human GMP synthetase. Further included are recombinant processes for the production of human GMP synthetase. The invention also includes nucleic acid sequences coding for human GMP synthetase, expression vectors comprising this coding sequence and host cells transformed with these expression vectors capable of expressing human GMP synthetase. Also included are polynucleotide probes capable of hybridizing to nucleic acid sequences of human GMP synthetase and assays for human GMP synthetase using such probes. Also included are antibodies to human GMP synthetase, assays for GMP synthetase using such antibodies and assays for inhibitors of human GMP synthetase activity using the recombinant or purified natural enzyme disclosed by this invention. The invention also includes inhibitors of human GMP synthetase preferably having an IC_{50} of 5 μ M or less.

Before proceeding further with the description of the specific embodiments of the invention a number of terms will be defined.

The term "purified human GMP synthetase" refers to a human GMP synthetase which is essentially free, i.e. contains less than about 30%, preferably less than about 10%, and even more preferably less than about

95% of the proteins with which the human GMP synthetase is naturally associated. Techniques for assessing purity are well known to the art and include, for example, sodium dodecyl sulfate (SDS) gel electrophoresis, high pressure liquid chromatography (HPLC), isoelectric focusing and capillary gel electrophoresis. Thus, the term purified also refers to a human GMP synthetase which appears as essentially one band on SDS gel electrophoresis when visualized by Coomassie blue staining. Purity can alternatively be assessed by measurement of the activity of the human GMP synthetase, for example, by the rate at which the purified human GMP synthetase produces adenosine 5'-monophosphate (AMP). Thus, the term purified may also refer to a human GMP synthetase that produces AMP at a rate at least 100 fold faster than, preferably 500 fold faster than, and more preferably 900 fold faster than, the human GMP synthetase in its naturally occurring state when measured under the conditions disclosed herein. The term purified can also refer to a human GMP synthetase that has a specific activity of at least 2.5 μ moles (2,500,000 pmoles) of AMP produced/min/mg when enzyme activity is measured using the conditions described in Example 1. The activity of the human GMP synthetase is measured by determining the rate at which it catalyzes the conversion of XMP to GMP or ATP to AMP under the conditions described in the Examples 1 and 3.

The term " IC_{50} " refers to the concentration of a compound that is required to inhibit by 50% the activity of purified human GMP synthetase when enzyme activity is measured under the conditions of the procedure # 2, the radioactive assay, described in Example 5. Briefly, the assay measures the conversion of XMP to GMP or ATP to AMP in the presence or absence of a suspected inhibitory compound. Therefore, the concentration of the inhibitory compound in the assay that reduces the amount of GMP or AMP produced by 50% compared to the amount of GMP or AMP produced in the absence of the compound is the IC_{50} of that compound.

The term "polynucleotide" as used herein refers to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. This term refers only to the primary structure of the molecule. Thus, this term includes double and single stranded DNA, as well as double and single stranded RNA. It also includes modified, for example, by methylation and/or by capping, and unmodified forms of the polynucleotide.

The term "recombinant polynucleotide" as used herein refers to a polynucleotide of genomic, cDNA, semisynthetic or synthetic origin which, by virtue of its origin or manipulation: (1) is not associated with all or a portion of the polynucleotide with which it is associated in nature and/or (2) is linked to a polynucleotide other than that to which it is linked in nature.

The term "cDNA" or complementary DNA refers to single stranded or double stranded DNA sequences obtained by reverse transcription of

messenger RNA isolated from a donor cell. For example, treatment of messenger RNA with a reverse transcriptase such as AMV reverse transcriptase or M-MuLV reverse transcriptase in the presence of an oligonucleotide primer will furnish an RNA-DNA duplex which can be treated with RNase H, DNA polymerase and DNA ligase to generate double stranded cDNA. If desired, the double stranded cDNA can be denatured by conventional techniques such as shearing to generate single stranded cDNA.

An "expression vector" is any genetic element, e.g., a plasmid, a chromosome, a virus, behaving either as an autonomous unit of polynucleotide replication within a cell (i.e. capable of replication under its own control) or being rendered capable of replication by insertion into a host cell chromosome, having attached to it another polynucleotide segment, so as to bring about the replication and/or expression of the attached segment. Suitable vectors include, but are not limited to, plasmids, bacteriophages and cosmids. Vectors will contain polynucleotide sequences which are necessary to effect ligation or insertion of the vector into a desired host cell and to effect the expression of the attached segment. Such sequences differ depending on the host organism; they include promoter sequences to effect transcription, enhancer sequences to increase transcription, ribosomal binding site sequences and transcription and translation termination sequences.

The term "transfer vector" refers to a plasmid that enables the integration of a recombinant gene into virus DNA by homologous recombination.

The term "host cell" generally refers to prokaryotic or eukaryotic organisms and includes any transformable organism which is capable of expressing a protein and can be, or has been, used as a recipient for expression vectors or other transfer DNA.

The term "transformed" refers to any known method for the insertion of foreign DNA sequences into a host cell whereby the inserted DNA is rendered capable of replication in the host cell. The transformation procedure depends on the host cell being transformed. It can include packaging the polynucleotide in a virus as well as direct uptake of the polynucleotide. Transformation can result in incorporation of the inserted DNA into the genome of the host cell or the maintenance of the inserted DNA within the host cell in plasmid form. Methods of transformation are well known in the art and include, but are not limited to, viral infection, electroporation, lipofection and calcium phosphate mediated direct uptake.

It is to be understood that this invention is intended to include other forms of expression vectors, host cells and transformation techniques which serve equivalent functions and which become known to the art hereto.

The term "cDNA library" refers to a collection of host cells transformed with cDNA sequences derived from a donor cell.

The term "reporter molecule" refers to a chemical entity capable of being detected by a suitable detection means, including, but not limited

to, spectrophotometric, immunochemical, or radiochemical means.

The term a polynucleotide "derived from" a designated sequence, for example, the GMPS.6 sequence depicted in Figure 1, which includes the cDNA of human GMP synthetase, refers to a polynucleotide sequence which is comprised of a sequence of approximately at least 6 nucleotides, preferably at least 8 nucleotides, more preferably at least 10-12 nucleotides, and, even more preferably, at least 15-20 nucleotides corresponding to, i.e., homologous or complementary to, a region of the designated sequence. The derived polynucleotide sequence is not necessarily physically derived from the nucleotide sequence shown, but may be derived in any manner, including for example, chemical synthesis or DNA replication or reverse transcription, which are based on the information provided by the sequences of bases in the region(s) from which the polynucleotide is derived.

The term "inhibitor" refers to a compound or extract which decreases the enzyme activity of human GMP synthetase when enzyme activity is measured in the presence of the inhibitor relative to the enzyme activity measured in the absence of the inhibitor. Specific methods of measuring enzyme activity in the presence of suspected inhibitors are described more fully in Example 5.

Purification of human GMP synthetase

The general method of purifying human GMP synthetase by the method of this invention comprises the steps of cell lysis, homogenization, centrifugation, anion exchange chromatography, hydrophobic interaction chromatography, and high resolution anion exchange chromatography.

Typically, a human cell line such as, for example, a T-lymphoblastoma cell line, a Jurkat cell line, or a B cell line expressing GMP synthetase is cultured under conditions appropriate to the particular cell line being used in an appropriate aqueous medium. The invention is not restricted to any particular types of human cell lines although transformed cell lines may provide better results than tissue. The medium will usually be a salt medium such as normal saline, phosphate buffer, or phosphate buffered saline containing additives well known to those skilled in the art and commercially available from a number of suppliers such as GIBCO and Hyclone. The cultured cells are then homogenized under conventional conditions such as, for example, sonication, freeze-thawing, and hypotonic lysis, and the cytosolic fraction is obtained by centrifugation and recovery of the supernatant. Differential salt fractionation, using, for example, ammonium sulfate, is employed to separate the GMP synthetase-rich protein fraction. Salt is then removed by one of a variety of known methods such as gel filtration, dialysis and the like. The desalted protein fraction is then subjected to anion exchange chromatography. This is preferably done using DEAE-cellulose or Q-Sepharose as examples of anion exchange material. Fractions showing GMP-synthetase activity are further subjected to hydrophobic interaction chromatography and a second

purification via anion exchange chromatography. GMP synthetase activity is measured by the spectrophotometric-coupled assay described by Spector (T. Spector; Methods Enzymology, 51, 219-224, (1978)). The assay measures the rate of production of AMP by GMP synthetase by coupling it to the oxidation of NADH via the auxiliary enzymes AMP kinase, pyruvate kinase and lactate dehydrogenase. In this fashion a purified human GMP synthetase is obtained. It has a molecular weight of approximately 75 kDa and is a single band on SDS gel electrophoresis when visualized by Coomassie blue staining. Furthermore, it has a specific activity of 2.5 μ moles AMP produced/min/mg or greater, often in the range 2.5 to 4.0 μ moles AMP produced/min/mg, in the spectrophotometric-coupled Spector assay referenced above.

Recombinant human GMP synthetase

As described above, the present invention provides purified human GMP synthetase. Purified human GMP synthetase may be used to design DNA probes for obtaining genomic or cDNA clones for use in the recombinant production of human GMP synthetase. For example, the amino acid sequence of purified protein can be determined by chemical techniques and oligonucleotide sequences coding for specific amino acid segments can be derived. Alternatively, the purified protein can first be subjected to enzymatic digestion using proteases such as trypsin, pepsin and the like to obtain peptide fragments whose amino acid sequences can be determined by techniques such as Edman degradation.

These amino acid sequences are used to construct oligonucleotide primers of complementary sequences which may be used in a polynucleotide amplification technique such as the polymerase chain reaction (PCR) (R.K. Saiki, D.H. Gelfand, S. Stoffel, S.J. Scharf, R. Higuchi, G.T. Horn, K.B. Mullis, and H.A. Ehrlich, Science, 239, 487-491 (1989)) to amplify cDNA from a human cell line to obtain a DNA sequence included within the gene coding for human GMP synthetase. Due to the degeneracy of the genetic code, oligonucleotide primers of complementary sequences are customarily prepared in all possible variations. On occasion, variation of the primer sequences may be limited based on homology with related enzymes and/or species specific codon usage. The identity of the DNA sequence obtained via amplification can be verified by subcloning into a second vector and sequencing positive clones to establish the presence of sequences corresponding to the originally used oligonucleotide primers. This DNA sequence obtained as above can then be used to screen a cDNA library obtained from a human cell line expressing GMP synthetase to identify the complete nucleic acid sequence coding for the enzyme.

The basic molecular biology techniques employed in accomplishing features of this invention, such as RNA and DNA and plasmid isolation, restriction enzyme digestion, preparation and probing of a cDNA library, sequencing clones, constructing expression vectors, transforming cells, maintaining and growing cell cultures and other general techniques are well

known in the art, and descriptions of such techniques can be found in general laboratory manuals such as Molecular Cloning: A Laboratory Manual by J. Sambrook, E.F. Fritsch and T. Maniatis, published by the Cold Spring Harbor Laboratory Press, 2nd edition, 1989.

5 The DNA sequences contemplated by this invention comprise oligodeoxynucleotides coding for human GMP synthetase as well as fragments thereof, as well as all equivalent nucleotide sequences coding for molecules with substantially the same human biological activity as human GMP synthetase. In addition to the polymerase chain reaction described
10 herein, preparation of such DNA sequences is also possible by other amplification techniques as well as known synthetic procedures.

A DNA sequence coding for human GMP synthetase can be cloned into a variety of expression vectors. The expression vectors include, for example, plasmids, bacteriophages and cosmids. The particular vector
15 chosen should be compatible with the contemplated host cell, whether a bacterium such as, for example, *E. coli*, or yeast or other cell. A plasmid should have the proper origin of replication for the particular host cell to be employed and suitable restriction sites that allow the ligation of foreign DNA sequences. Furthermore, the plasmid should impart to the
20 transformed cell a phenotypic property that will enable the transformed cells expressing the enzyme to be readily identified from cells that do not undergo transformation. Similar considerations apply to nonplasmid vectors. Numerous expression vectors with a variety of properties are available from commercial suppliers.

25 The DNA encoding the desired human GMP synthetase, whether in fused or mature form, and whether or not containing a signal sequence to permit secretion, is ligated into an expression vector suitable for any convenient host. Both eukaryotic and prokaryotic host cells are presently used in forming recombinant proteins. By way of illustration and not limitation,
30 eukaryotic systems include human fibroblast HeLa cells and Jurkat T-cells and prokaryotic systems include *E. coli*.

The expressed enzyme is then isolated from lysed cells or from culture medium and purified to the extent needed. Purification is carried out by methods known to the art, including, but not limited to, salt
35 fractionation, ion exchange chromatography, hydrophobic chromatography, gel filtration chromatography, affinity chromatography, isoelectric focusing and centrifugation.

The recombinant human GMP synthetase contemplated by this invention includes all functionally equivalent enzymes obtained by expressing in a
40 host cell a DNA sequence encoding for human GMP synthetase or a fragment thereof, as well as functionally equivalent sequences coding for human biologically active GMP synthetase.

In a preferred embodiment of this invention as described in more detail in Example 4, a DNA coding sequence for human GMP synthetase is
45 subcloned into a baculovirus transfer vector. Insect cells are co-

-11-

transfected with the transfer plasmid and baculovirus virion DNA. A few days later, when polyhedra are evident in the culture, the culture fluid is harvested and several clones of the recombinant virus plaque are purified. Expression levels of GMP synthetase of each of the recombinant viruses are determined by infecting insect cells and performing enzyme assays on the lysates of the infected cells. One of the clones with a high level of expression is selected for large scale production of the enzyme, wherein insect cells growing in serum free culture fluid are infected with the recombinant baculovirus obtained above. Sometime later the cells are harvested by centrifugation. The cells are suspended in hypotonic buffer and mechanically disrupted. Following clarification by centrifugation, the soluble proteins are fractionated by ammonium sulfate precipitation. The GMP synthetase containing fraction is desalted and the GMP synthetase is purified by sequential anion-exchange and hydrophobic interaction chromatography. A final purification using high resolution anion-exchange chromatography results in a purified preparation of GMP synthetase. The purified recombinant human GMP synthetase is a single band on SDS gel electrophoresis as visualized by Coomassie blue staining and has a specific activity of 3.0 $\mu\text{mol AMP produced/min/mg}$ or greater, often in the range 3.0 to 4.4 $\mu\text{mol AMP produced/min/mg}$, when measured by the spectrophotometric-coupled Spector assay referenced earlier.

Assays for inhibitors of human GMP synthetase

The present invention also relates to assays using purified human GMP synthetase to screen for and identify inhibitors of human GMP synthetase. The assay is a method comprising contacting a medium containing the suspected inhibitor with purified human GMP synthetase and measuring the activity of the human GMP synthetase. The medium can be an extract of biological origin, such as, for example, a plant, animal, or microbial cell extract, or alternatively, it may be a substantially pure compound of synthetic origin, or a mixture of compounds thereof.

Enzyme activity can be measured by methods such as direct measurement of the products of the enzymatic reaction or by coupling the formation of the reaction product to the reaction of other detectable species. For example, in one method for determining enzyme activity the formation of AMP and/or GMP is measured by coupling their formation to the oxidation of NADH as mediated via AMP kinase and/or GMP kinase, pyruvate kinase, and lactate dehydrogenase. The oxidation can be monitored spectrophotometrically at 340 nm. In another method for measuring the enzyme activity, radiolabeled XMP or ATP is used as substrate and the formation of radioactive GMP or AMP is measured directly after separation of substrates and products by chromatographic methods such as thin layer chromatography or high pressure liquid chromatography.

Compounds are identified based on their ability to inhibit the activity of the purified human GMP synthetase disclosed herein. The assay is used to identify compounds having an IC_{50} of less than 25 μM , preferably,

less than 20 μM , more preferably, less than 10 μM . The preferred inhibitors have an IC_{50} greater than 0 and less than 5 μM . Usually, the present inhibitors have an IC_{50} of less than 5 μM , preferably less than 1 μM , generally 0.01 to 5 μM , more preferably 0.1 to 1 μM . The inhibitors are used in a method for inhibiting human GMP synthetase activity by administering an effective amount of the inhibitor having an IC_{50} of 5 μM or less. The effective amount is generally that amount that results in greater than 50% decrease in human GMP synthetase activity, preferably, greater than 70% decrease in human GMP synthetase activity, more preferably, greater than 90% decrease in human GMP synthetase activity.

Antibodies against human GMP synthetase

The present invention also relates to antibodies, both polyclonal and monoclonal, against human GMP synthetase. Methods of preparing polyclonal antibodies are well known to the art. For example, an immunogenic conjugate comprising the human GMP synthetase or a fragment thereof, optionally linked if necessary to a carrier protein, is used to immunize a selected mammal (e.g. mouse, rabbit, goat etc.). Serum from the immunized mammal is collected and treated according to known procedures to separate the immunoglobulin fraction. In one approach in the present invention, the human GMP synthetase is expressed as a fusion transfer protein, such as, for example, glutathione transferase fusion protein, which is used to immunize rabbits according to a conventional procedure.

Monoclonal antibodies are prepared by standard hybridoma cell technology based on that reported by Kohler and Milstein in *Nature* 256 (1975) 495-497. Briefly, spleen cells are obtained from a host animal immunized with human GMP synthetase protein or a fragment thereof, optionally linked to a carrier if necessary. Hybrid cells are formed by fusing these spleen cells with an appropriate myeloma cell line and cultured. The antibodies produced by the hybrid cells are screened for their ability to bind to purified human GMP synthetase. A number of screening techniques well known in the art, such as, for example, forward or reverse enzyme-linked immunosorbent assay (ELISA) screening methods may be employed. The hybrid cells producing such antibodies are then subjected to recloning and high dilution conditions in order to select a hybrid cell that secretes a homogeneous population of antibodies specific to human GMP synthetase. The antibodies of this invention find use in the detection of human GMP synthetase.

The antibodies of this invention can be conjugated to a reporter molecule by techniques well known in the art. Typically the reporter molecule contains a functional group suitable for attachment of the reporter group to the antibody. The functional groups suitable for attaching the reporter group are usually activated esters or alkylating agents which react with nucleophilic groups such as, for example, amino, hydroxyl or thiol groups on the antibody. Details of these and related techniques for attaching reporter groups to proteins are described in

reviews such as, for example, Enzyme-Immunoassay by E.T. Maggio, published by the CRC Press, Boca Raton, FL, 1980.

Generation of Nucleic Acid Probe

Purified human GMP synthetase is subjected to peptide digestion, using, for example, trypsin, pepsin, or other proteases and the like, and the peptides are resolved by chromatographic techniques such as, for example, reverse phase high pressure liquid chromatography. The peptides are sequenced. Degenerate oligonucleotides are synthesized corresponding to the derived amino acid sequences of the above peptides. Oligonucleotides are synthesized in both the sense and antisense directions for each peptide and are used as primers in an amplification by the polymerase chain reaction. Since the location of the peptides in the protein sequence is unknown, each oligonucleotide is used with another as primers. A template for the PCR reaction is constructed by generating the cDNA complementary to RNA, such as polyadenylated RNA, from a human cell line expressing GMP synthetase. This is accomplished by extracting the total cellular RNA from the cell line of interest, separating the RNA fraction of interest by column chromatography using the appropriate column substrate, such as, for example, in the case of polyadenylated RNA, an oligo-dT cellulose column, and treating it with reverse transcriptase. A DNA amplification reaction is carried out by repeated primer initiated strand extension and fragments are generated and isolated by appropriate techniques. Polynucleotide probes are prepared by conjugation of reporter molecules to fragments isolated as above. Identification of DNA sequence encoding for human GMP synthetase

The fragments obtained above are used to screen a cDNA library prepared by using RNA from human cells with a suitable cDNA cloning vector. A wide variety of cloning vectors are available, including lambda vectors and phagemid vectors. Second generation "Okayama-Berg" vectors are also available for efficient cDNA cloning and subsequent expression in mammalian systems. In one such instance using polyadenylated RNA, a number of positive clones are isolated of which the four longest are overlapping. The longest sequence, GMPS.6 (SEQ ID NO: 1), is shown in Figure 1 and was approximately 2.2kb. In Figure 1, amino acid residues are represented in single-letter code. Lines and numbers in parentheses mark the position of sequences corresponding to the tryptic peptides determined from the purified native enzyme. Boxes mark the position of sequences corresponding to the oligonucleotide primers. Nucleotide residue 1 represents the start site of translation.

Sequence analysis of GMPS.6 (SEQ ID NO: 1) reveals an open reading frame of 2079 base pairs (Figure 1). This open reading frame yields a protein of 693 amino acid residues and a predicted molecular weight of 76,725. This predicted size is in good agreement with the 75kDa size of the purified human GMP synthetase from natural sources obtained above. The sequences of all nine tryptic peptides are contained within the predicted

protein sequence and are shown in Table 2 in Example 2.

Expression of recombinant human GMP synthetase

One embodiment of the invention is exemplified by a recombinant protein prepared by subcloning the cDNA sequence spanning bases 1 to 2090 of GMPS.6 (SEQ ID NO: 1) into the NcoI/Pst I cloning site of the prokaryotic expression vector, pTRC.99A (Pharmacia) which contains an ampicillin resistance marker and the trc promoter that is inducible by IPTG. This plasmid is designated NF.6 (NF designates "no fusion") and contains the entire open reading frame and all of the 3'-untranslated sequence, but none of the 5'-untranslated sequence. A host cell is transfected with the NF.6 plasmid and cultured to express the recombinant protein. Purification of the recombinant protein yields a product of greater than 95% of purity as determined by SDS gel electrophoresis and Coomassie blue staining. The recombinant enzyme of this embodiment has the identical kinetic and biochemical properties as the purified human enzyme described previously.

Functional expression of human GMP synthetase in this fashion is further verified by testing for complementation of guanosine requirement in AT2465 E.coli transfected cells which do not grow in absence of guanosine. A second expression vector is constructed as described above except that two stop codons are inserted into the ligated oligonucleotide 39 base pairs downstream from the start site of the translation. This expression vector is designated ES.6 (ES abbreviates "early stop") and it coded for a truncated protein, as expected. Both ES.6 and NF.6 are transfected into AT2465 E. coli, and the cells are allowed to grow on minimal medium plates containing ampicillin and IPTG with or without guanosine. As shown in Figure 2, cells that are not transfected do not produce colonies on either plate due to the absence of ampicillin resistance in the parental AT2465 cells. Cells that are transfected with either NF.6 or ES.6 produce colonies in the presence of guanosine. Only cells transfected with NF.6 form colonies in the absence of guanosine. This result shows that GMPS.6 (SEQ ID NO: 1) contains a cDNA that can complement a host E. coli that was deficient in GMP synthetase. When this cDNA is mutated in ES.6 by the insertion of stop codons, it can no longer complement growth in the absence of guanosine. These results show complementation by the transfected human DNA.

The transfected cells also show the presence of GMP synthetase enzyme activity. Single colonies are selected from plates without guanosine (NF.6 transfected cells) or with guanosine (ES.6 transfected cells). As shown in Figure 2, GMP synthetase activity is present in NF.6 transfected cells and absent in ES.6 transfected cells. Furthermore, the activity is induced by the presence of IPTG. There is no detectable GMP synthetase activity in untransfected cells. These findings confirm the complementation results and demonstrate the cloning of a cDNA encoding a functional human GMP synthetase.

EXAMPLES

Materials

Restriction endonucleases and all enzymes used for plasmid construction were purchased from New England Biolabs (Beverly, MA); oligo (dT)-cellulose from Pharmacia LKB (Uppsala, Sweden); Taq DNA polymerase from Perkin Elmer Cetus (Norwalk, CT); [α - 32 P]dCTP from Amersham (Arlington Heights, IL); [α - 32 P]UTP from Du Pont NEN (Boston, MA). All other chemicals were purchased from Sigma (St. Louis, MO).

Cells and Media

A3.01 cells were obtained from Dr. Thomas Folks (National Institute of Health, Bethesda, MD. Folks, T., Benn, S., Rabson, A., Theodore, T., Hoggan, M. D., Martin, M., Lightfoote, M., and Sell, K. (1985) *Proc. Natl. Acad. Sci. USA* 82, 4539-4543). The A3.01 cells were cultured in RPMI 1640 media containing 5 % fetal bovine serum and 10 μ g/ml gentamicin (Hyclone, Logan, UT). CEM, HL60, U937 and WI38 cells were purchased from American Type Culture Collection (Rockville, MD); Jurkat and UC cells were from Dr. Jeffrey Northrop (Stanford University Medical Center, Stanford, CA); VB and HFF cells were provided by the cell culture facility at Syntex Corporation (Palo Alto, CA). Normal peripheral T lymphocytes were isolated from a healthy donor according to an approved protocol and purified as described by Eugui, E. M., and Almquist, S. J. (1990) *Proc. Natl. Acad. Sci. USA* 87, 1305-1309.

The bacterial hosts, SURE and XL1-Blue, used in library amplification and screening, were provided by Stratagene (La Jolla, CA). The AT2465 strain of *E. coli* was obtained from the *E. Coli* Genetic Stock Center (Yale University, New Haven, CT). The AT2465 cells have the following chromosomal markers: thi-1, guaA21, relA1, λ and spoT1. AT2465 cells do not grow in minimal media in the absence of guanosine and thiamin (Taylor, A. L., and Trotter, C. D. (1967) *Bacteriol. Rev.* 31, 332-353 and references therein).

EXAMPLE 1

Purification of Naturally Occurring Human GMP Synthetase

A particularly preferred method of purification of naturally occurring human GMP synthetase from A3.01 cells is described below.

Step 1: Homogenization. In a typical purification, A3.01 cells were cultured in RAMI 1640 media containing 5% fetal bovine serum and 10 μ g/ml gentamicin (Hyclone, Logan, UT). The cells were lysed in 15 ml of buffer A (20 mM Tris.HCl, pH 7.6, 0.1 mM DTT, 0.5 mM EDTA, 10% glycerol) by the use of a glass teflon homogenizer. The homogenate was centrifuged at 15,000 x g for 20 min and the pellet was discarded. Purification was carried out with the supernatant (cytosol).

Step 2: Ammonium Sulfate Fractionation. Ammonium sulfate was added to the cytosol fraction obtained above until 35% saturation and the precipitated proteins were removed by centrifugation at 20,000 x g for 20

-16-

min and discarded. The proteins in the supernatant were precipitated with further addition of ammonium sulfate (60% saturation). These precipitated proteins, which include GMP synthetase, were recovered by centrifugation as above. The protein pellet was dissolved in 12 ml buffer A. Ammonium sulfate was removed from the protein solution by gel filtration chromatography on PD-10 columns (Pharmacia).

Step 3: DEAE-Cellulose Chromatography. The desalted protein fraction obtained above was applied to a 1.5 x 7.3 cm column of DEAE-cellulose (Whatman) equilibrated in buffer A. The enzyme was eluted with a 60-ml gradient of 0 to 0.5M sodium chloride in buffer A. The fractions containing GMP synthetase activity were pooled.

Step 4: Phenyl-5PW Chromatography. The DEAE cellulose pool obtained above was diluted with an equal volume of 0.2M potassium phosphate, pH 7.0. 1.7M ammonium sulfate, 20% glycerol. The protein sample was applied to a 8 mm x 7.5 cm column of Phenyl-5PW (ToscoHaas, Montgomeryville, PA) equilibrated in buffer B (0.1M potassium phosphate, pH 7.0, 1.7M ammonium sulfate, 10% glycerol). GMP synthetase was eluted with a 40-ml gradient of 1.7 to 0M ammonium sulfate in buffer B and the fractions containing active enzyme were pooled.

Step 5: Mono-Q Chromatography. The Phenyl-5PW pool obtained above was desalted as above and applied to a Mono Q HR 5/5 column (Pharmacia) equilibrated with buffer C (20 mM Tris. HCl, pH 8.0, 0.1 mM DTT, 20% glycerol). GMP synthetase was eluted with a 30-ml gradient of 0-0.3M sodium chloride in buffer C. The GMP synthetase fractions that contained a single Coomassie blue-staining band at 75kDa were pooled. This material is purified human GMP synthetase.

A purification table from a representative purification for the various steps of the process is shown in Table 1 below. Enzyme activity was determined by using the spectrophotometric-coupled assay of Spector performed exactly as described (T. Spector; Methods Enzymology, 51, 219-224, (1978)), incorporated herein by reference. This assay measured the rate of AMP production by GMP synthetase, and the level of AMP is measured through the activity of three auxiliary enzymes. When crude enzyme samples such as those in steps 1 to 3 of the purification were examined, this assay method measured a high rate of AMP formation even in the absence of the GMP synthetase substrate, XMP. This rate is referred to as background rate. The GMP synthetase activity shown in Table 1 below was the rate of AMP formation in the presence of all the substrates subtracted by the background rate when XMP is omitted. The GMP synthetase activity determined by this assay was the same as the activity determined by measuring radioactive GMP formation as described below in Example 3.

-17-

TABLE 1

5	sample	total protein	total activity	specific activity	fold purified
		(mg)	(μ mol AMP/min)	(μ mol AMP/min-mg)	
	cytosol	2478.55	7.42	0.0030	1
	35-60% AS precipitate	1095.46	10.24	0.0094	3
10	DEAE-cellulose pool	46.64	3.25	0.0670	23
	Phenyl-SPW pool	3.95	1.59	0.4038	135
	Mono Q pool	0.17	0.47	3.0	936

15

Figure 3 shows the SDS polyacrylamide gel electrophoresis of each step in the purification. Samples from each step of the purification were subjected to SDS polyacrylamide gel electrophoresis and stained with Coomassie blue. The purification step and amount of protein loaded in each lane are as follows: lanes 1. 35-60 % ammonium sulfate precipitate, 6 μ g; 2. DEAE-cellulose pool, 2.5 μ g; 3. Phenyl-5PW pool, 3 μ g; 4. Mono Q pool, 3 μ g. The purified human GMP synthetase was essentially one band after the final purification step.

25 The above purification was repeated on several occasions and samples of human GMP synthetase with specific activities in the range of 2.5-4.0 μ mol AMP produced/min/mg were routinely obtained.

30 EXAMPLE 2

Generation of Nucleic Acid Probes and Identification of the Gene for Human GMP Synthetase

GMP synthetase from A3.01 cells was purified to homogeneity as described above and digested with trypsin. Nine tryptic peptides were resolved by reverse-phase high-pressure liquid chromatography (RP-HPLC) and subjected to sequence analysis by Edman degradation using an ABI470A sequencer (Applied Biosystems, Foster City, CA). The sequences of the nine peptides are shown in Table 2 below. The sequences are represented by the standard one-letter code. The letter "X" indicates that no amino acid can be assigned from the corresponding cycle of Edman degradation.

45

-18-

TABLE 2

Peptide	Sequence
1	NIVAGIANESK (SEQ ID NO: 2)
2	ALNQEQVIAVXIDNGFM (SEQ ID NO: 3)
5 3	VINAASHFYNGTXKLPSDED (SEQ ID NO: 4)
4	IIGDTFV (SEQ ID NO: 5)
5	ANEVIGXNNLK (SEQ ID NO: 6)
6	VIEPLKDFIKDEVR (SEQ ID NO: 7)
7	PPPGGLAI (SEQ ID NO: 8)
10 8	FITSDFMGTGIPATPGNEIKV (SEQ ID NO: 9)
9	IMYDLTSKPPGTTE (SEQ ID NO: 10)

Based on the peptide sequences, degenerate oligonucleotides were synthesized using the phosphoramidite method with a Cyclone Plus DNA Synthesizer (MilliGen/Bioscience, Burlington, MA). Oligonucleotides were synthesized in both the sense and antisense orientations for each peptide and used as primers in polymerase chain reactions (PCR). To generate the template, DNA complementary to A3.01 polyadenylated RNA was prepared using avian reverse transcriptase (Life Sciences) using the methods disclosed by Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. DNA amplification was performed, using *Thermus aquaticus* (Taq) polymerase in a Perkin-Elmer/Cetus DNA thermal cycler using the methods disclosed in Heller, R. A., Song, K., Onasch, M. A., Fischer, W. H., Chang, D., and Ringold, G. M. (1990), *Proc. Natl. Acad. Sci. USA*, 87, 6151-6155. A specific fragment of approximately 1-kb in length was generated only when oligonucleotides 2S² and 8A² (corresponding to tryptic peptides 2 and 8) were used as primers (Figs 1 and 4). To confirm the identity of this fragment, it was subcloned into pCR.1000 (Invitrogen, San Diego, CA). From partial sequence analysis, the sequences corresponding to 2S and 8A at the 5' ends of the forward and reverse strands respectively, as well as the sequence of oligonucleotide 3S² (corresponds to peptide 3) near the 5' end of the forward strand (see Figures 1 and 4) were detected. This PCR fragment (pCR.2S8A) was used to screen an A3.01 cDNA library. Based on subsequent sequence analysis of GMPS.6, (SEQ ID No: 1) the sequence of pcr.2S8A corresponds to the sequence within Figure 1 starting from residue 781 and ending at residue 1952.

Screening of cDNA Library and Sequencing of Positive Clones

A cDNA library, prepared by Stratagene (La Jolla, CA), was constructed in the Lambda-ZAP II vector system using polyadenylated RNA from A3.01 cells. To obtain polyadenylated RNA, total cellular RNA was isolated according to the method of Chirgwin et al (Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979) *Biochemistry* 18, 5294-5299) and polyadenylated RNA was then obtained using oligo(dT)-cellulose as described by Aviv et al (Aviv, H., and Leder, P. (1972) *Proc.*

Natl. Acad. Sci. USA 69, 1408-1412). The resulting cDNA library prepared by Strategene yielded 9.3×10^6 primary plaques with the host bacterial strain SURE (Stratagene, La Jolla, CA). Amplification was performed on 1×10^6 plaque forming units (pfu).

The amplified library was screened according to a standard procedure provided by Strategene. The PCR fragment pcr.2S8A was radiolabeled by random-hexamer priming and it was used as a probe. A total of 0.9×10^6 pfu were screened. After three rounds of screening, eight positive phagemids were excised and rescued by R407 helper phage (Stratagene). Doubled stranded pBluescript (SK) plasmids were isolated, and four of the longest clones were analyzed further by restriction mapping and found to be overlapping.

The cDNA inserts of the four positive clones were sequenced by the method of Sanger using fluorescent dye-labeled terminators with a 373A DNA Sequencer (Applied Biosystems). Partial sequence analysis confirmed that the four clones were overlapping. The complete sequence of both strands of the insert of clone 6 (GMPS.6, SEQ ID NO: 1) was determined and is shown in Figure 1. Sequence analysis of GMPS.6 revealed an open reading frame of 2079 base pairs (Figure 1). The first ATG was found at 142 bases downstream from the 5' end and a stop codon TAA was found 11 bases upstream from the 3' end. No poly(A) tail was found on any of the clones. The open reading frame yielded a protein of 693 amino acid residues with a predicted molecular weight of 76,725. The derived amino acid sequence (SEQ ID NO: 11) of human GMP synthetase is shown in Figure 1. The predicted size of the enzyme was in good agreement with the 75-kDa size indicated by polyacrylamide gel electrophoresis of the purified A3.01 human GMP synthetase described in Example 1. The sequences corresponding to the nine tryptic peptides were located within the predicted peptide sequence.

EXAMPLE 3

Construction of Expression Plasmids and Complementation in AT2465 Cells

The insert of GMPS.6 was excised from the pBluescript plasmids obtained above and cloned into the *Nco* I / *Pst* I cloning site of the prokaryotic expression vector, pTRC99A (Pharmacia), to yield vector TRC.6. Using TRC.6, two expression vectors, NF.6 and ES.6, were constructed to remove the 5'-untranslated sequence of the cDNA. A 201-bp fragment was removed from the 5' end of the TRC.6 by digestion with *Nco* I and *Msc* I, and replaced with a 60 base pair-oligonucleotide that had the identical sequence of residues -1 to 59 except for a G to C change at residue -1 (see Figure 1, residue 1 represents the start site of translation). This base change converted the *Bgl* I site at the start site of translation to an *Nco* I site. The 60 base pair-oligonucleotide which has the sequence 5'-CATGGC... was directly ligated into the *Nco* I/*Msc* I cut vector, and this expression vector was designated NF.6 (NF abbreviates "no fusion"). A second expression vector ES.6 was constructed in the same manner except that two stop codons were inserted in the ligated oligonucleotide 39 base

-20-

pairs downstream from the start site of translation (ES abbreviates "early stop"). With reference to Figure 1, the nucleotide sequence from residues 40 to 45 was changed from **GGAGGA** to **TGATA**.

To test for complementation of the guanosine requirement, AT2465 cells that were transfected with NF.6 or ES.6 were spread on M9 minimal media plates with or without guanosine (100 µg/ml). All plates were supplemented with 0.4% glucose, 0.2% casamino acids, 20 µg/ml IPTG and 100 µg/ml of: thiamin, glutamine, histidine, arginine, inosine, biotin, 2'-deoxyuridine and ampicillin. Colony formation was examined after 16-48 h of growth.

Cells that were not transfected did not produce colonies on either plate due to the absence of ampicillin resistance in the parental AT2465 cells (Fig. 2). Cells that were transfected with either NF.6 or ES.6 produced colonies in the presence of guanosine. Only cells transfected with NF.6 formed colonies in the absence of guanosine. This result showed that GMPS.6 contained a cDNA that could complement a host *E. coli* that was deficient in GMP synthetase. When this cDNA was mutated in ES.6 by the insertion of stop codons, it could no longer complement growth in the absence of guanosine. These data show complementation by the transfected human DNA.

To determine GMP synthetase activity, single colonies were selected and inoculated in LB media containing 100 µg/ml ampicillin. Cultures were allowed to grow to mid-log phase and then IPTG was added to a final concentration of 20 µg/ml. Cells were harvested 3 h after the addition of IPTG.

Determination of Enzyme Activity in Transfected *E. coli* Cells

Cells were resuspended in lysis buffer (20 mM Tris-HCl, pH 7.6, 0.5 mM DTT, 0.2 mg/ml lysozyme, 2 mM PMSF, 0.5 mM EDTA, and 10% glycerol) and lysed at 37 °C for 10 min. Genomic DNA was sheared by passing the lysate through a 27 gauge needle three to four times. The cytosolic fraction was separated from the membrane fraction by centrifugation at 15,000 x g for 15 min. GMP synthetase activity in the cytosol was determined by measuring the formation of [8-¹⁴C]GMP from [8-¹⁴C]XMP (50 mCi/mmol, Moravsek, Brea, CA). The assay mixture contained 75 mM Tris-HCl, pH 7.8, 10 mM MgCl₂, 2 mM ATP, 1 mM [1-¹⁴C]XMP (10 mCi/mmol), 5 mM glutamine and 50 mM DTT. Typically, 5 µl of cytosol was added to 25 µl of assay mix, and the reaction was allowed to proceed at 40 °C for 15 min. The reaction was stopped by the addition of 6 µl of quench solution, which contained 250 mM EDTA and 62.5 mM each of GMP and XMP as carriers. GMP and XMP were separated by thin layer chromatography as described (Boritzki, T. J., Jackson, R. C., Morris, H. P., and Weber, G.; (1981) *Biochim. Biophys. Acta*, 658, 102-110). Briefly, the quenched reaction mixtures (10 µl) were streaked onto 2.5 cm x 20 cm strips of polyethyleneimine c llulose plates (Macherey-Nagel, Germany). The strips were developed in 2 M formic acid. The position of GMP was visualized by ultraviolet light and marked. The GMP band was excised, and

-21-

the amount of [¹⁴C]GMP was determined by liquid scintillation counting. Protein concentration was determined by Bradford analysis (Bio-Rad).

The results shown in Figure 2 demonstrate that GMP synthetase activity was present in NF.6 transfected cells and absent in ES.6 transfected cells. Furthermore, the activity was induced by the presence of IPTG. There was no detectable GMP synthetase activity in untransfected cells. These findings confirm the complementation results and demonstrate that the cloning of a cDNA encoding a functional human GMP synthetase.

EXAMPLE 4

Expression, Production and Purification of Recombinant Human GMP Synthetase Lipofection:

A lipid solution consisting of lipofectin (0.067 mg/ml) in HEPES Buffered Saline (HBS), (20 mM HEPES, 150 mM NaCl, pH 7.4) and a DNA solution containing 0.33 ug Autographa californica Nuclear Polyhedrosis Virus DNA (BaculoGold[®], Pharmingen, San Diego, CA) and 3.3 ug transplacement plasmid (pSYN-GMPs) DNA were prepared.

The transplacement plasmid, pSYN-GMPs, was prepared as follows. The coding region of the GMP synthetase cDNA was excised from NF.6 and cloned into the baculovirus expression vector pSynXIV VI*X3 (Wang, X., Ooi, B. G., and Miller, L. K. (1991) Gene 100, 131-137) to yield pSyn.GMPs. The vector pSynXIV VI*X3 was linearized at the EcoRI site in the multiple cloning region downstream from the promoter, and the 3'-recessed sequences were filled in using Klenow fragment as described in Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. The blunt-ended vector was digested with PstI which removed one blunt end. NF.6 was linearized with NcoI which cleaved NF.6 at the start site of translation. Blunt ends were created as described above and the vector was cut with PstI to release the coding region insert. The insert, which was blunted at the 5'-end and cleaved at the 3'-end with PstI, was cloned into the blunt/PstI cloning site of the pSynXIV VI*X3 vector to yield the pSYN-GMPs expression vector.

Plastic flasks (Costar, tissue culture, 25 cm² surface area) were seeded with 3 X 10⁶ Spodoptera frugiperda (Sf-9) cells (ATCC, Rockville, MD) that had been adapted to grow in suspension in ExCell-400 medium (JRH Scientific). After 1 hour the cells which had attached to the flask were washed with HBS and three milliliters of lipofection solution consisting of 1 part lipid solution and 1 part DNA solution were added to the monolayer. After 40 minutes at 28°C, three milliliters of ExCell-400 medium supplemented with 10% fetal calf serum and gentamicin (50 ug/ml) were added to the lipofection solution in the flask. Thirty minutes later the lipofection solution was decanted and replaced with fresh ExCell-400 medium supplemented with 2.5% fetal calf serum. Five days later, the culture fluid was collected (5 day harvest) and centrifuged at 2000 rpm for 15

-22-

minutes. Fresh Ex-Cell 400 medium supplemented with 2.5% FCS was added to the flask and a second harvest is taken 2 days later (7 day harvest).

Plaque Purification of Recombinant Baculovirus:

The clarified culture fluid from the transfected cells (either the day 5 or day 7 harvest) was serially diluted for 1×10^1 to 1×10^6 . Six-well plates (Linbro, tissue culture, 9.6 cm² surface area per well) were inoculated with 2 ml Sf-9 cell suspension (0.6×10^6 cells/ml ExCell-400). When the cells attached, 1 hour after seeding, each well was inoculated with 0.5 ml of each dilution of the culture fluid in duplicate. Viral adsorption continued for 1 hour at 28°C. The plates were rocked every 15 minutes to facilitate distribution of the virus particles. The inoculum was then aspirated, and the cell monolayers in each well overlaid with 2 ml of a solution containing 1 part 2X ExCell-400, 1 part 3% melted Sea-Plaque Agarose, and gentamicin (50 ug/ml). The plates were incubated in a humidified environment at 28°C for 5 days until plaques had developed. A second 2 ml overlay that contained 0.1 mg/ml Neutral Red (GIBCO Labs, Grand Island NY) as well as the components of the first overlay was added to each well. The following day, plaques were readily visible. When observed under a dissecting microscope, polyhedra could be seen at the center of the plaques. Plaques were picked with a P-200 Pippettman[®] with the volume adjusted to 100 ul and equipped with a sterile tip. Four well separated plaques were picked and dispensed into 0.5 ml fresh ExCell-400 medium. The virus from each plaque was repurified three times.

Preparation of Master Seed Virus:

After the third cycle of plaque purification, virus from a single plaque suspended in 0.5 ml ExCell 400 was inoculated into a well of a 6 well plate containing 1.2×10^6 cells. After 1 hour adsorption, 1.5 ml ExCell 400 medium was added to each plate. The plates were incubated at 28°C in a humidified environment for 4-5 days when polyhedra were evident in 70-80% of the cells. The culture fluid from each well was clarified by centrifugation at 1500 X g for 10 minutes. Once the culture fluid was titrated, a 100 ml Sf-9 Cell culture suspended in a 250 ml plastic Erlenmeyer shake flask at a density of 1.2×10^6 cells/ml was infected with the above virus. The ExCell 400 medium was supplemented with 1% BSA or 5% FCS and 4% feed solution. The feed solution consists of 1.25% glutamine, 2.5% glucose, 12.5X lipid concentrate (GIBCO-BRL, Grand Island, NY, cat. # 21900-004), and 12.5 X yeastolate ultrafiltrate (GIBCO-BRL, Grand Island, NY, cat # 18200-014). The multiplicity of infection was 0.5 plaque forming units per cell. The cells were maintained in suspension by shaking at 140 rpm in a rotary shaker at 28° C. Four days after infection, the culture fluid was harvested by centrifugation at 1500 X g for 10 minutes. The supernatant was collected and titrated. Aliquots were frozen at -80°C.

Preparation of Working Seed Virus Stock:

An aliquot of master seed virus was thawed and a 100 ml culture of the Sf-9 cells growing in ExCell-400 in a 250 ml plastic Erlenmeyer flask was infected at a MOI of 0.5. The medium was supplemented with either a 1% BSA or 5% FCS and 4% feed solution. The cells were incubated at 28°C at 140 rpm in a rotary shake flask for four days. The cells were then pelleted and the culture fluid was utilized as working seed passage 1. The virus was titered and stored at 4°C until needed. In order to increase the amount of virus needed for large scale production of human GMP synthetase working seed passage virus 1 is used to infect fresh Sf-9 cells as described above. The virus from this second round of infection is referred to as working seed passage 2. It is harvested as described above and stored at 4° until needed.

Sf-9 cells (1.2×10^6 /ml) growing in 2 liters of ExCell-400 medium in a 3 liter bioreactor (Applikon, Foster City, CA) were infected with working seed passage 1 virus at a multiplicity of infection (MOI) of 0.5. The cells were maintained in suspension by rotation of the impellers at 100-200 rpm. The medium, ExCell-400, was supplemented with 0.1% Pluronic F68, 50 ug/ml gentamicin and antifoam AF Emulsion (JRH Biosciences, Lenexa, Kansas) up to 10 ppm. The culture was also fed 80 ml feed solution at the time of infection. The pH was maintained at 6.3 by titration with 2N NaOH and the dissolved oxygen was maintained at 30% saturation by sparging with air and adjusting the impellar speed. After four days at 28° C, the cells were pelleted by centrifugation and the supernatant was titered and stored at 4°C until needed.

Production of Human GMP Synthetase:

Production of GMPS was carried out in 10 liters of ExCell-400 supplemented with 1% Pluronic F68. The cells were infected at a density of 1.2×10^6 cells per ml with working seed passage 2 at an MOI of 0.5. The cells were fed with 400 ml feed solution at the time of infection. The culture was maintained at 27°C and the pH adjusted to 6.3 with 2N NaOH, and 30% saturation dissolved oxygen for three days. The ExCell-400 medium was supplemented with gentamicin as described above. The cells were then harvested by centrifugation and frozen at -80 C until needed for purification.

Purification of GMP Synthetase:

Sf-9 cells obtained from above were suspended in lysis buffer (20 mM Tris pH 8.0, 0.5 mM EDTA, 0.5 mM DTT, 10% glycerol, 2 ug/ml leupeptin, 2 ug/ml pepstatin, 2 ug/ml aprotinin). After mixing for 10 minutes, the cells were transferred to a Dounce (type B) homogenizer and the cells disrupted with 20 strokes of the homogenizer. Nuclei and other cell debris were pelleted by centrifugation for 20 minutes at 41,000 X g. Ammonium sulfate was added to the supernatant slowly to give a 35% saturation solution. After stirring for 30 minutes, the preparation was subjected to centrifugation (41,000 X g, 20 minutes). The pellet was discarded and

additional ammonium sulfate added to the supernatant so that the final concentration was 60% of saturation. The mixture was stirred 30 minutes and then subjected to centrifugation (10,000 X g, 10 minutes). The pellet was resuspended in an equal volume of lysis buffer and transferred to a 6000-8000 mwco dialysis bag. The suspension is dialyzed against 10 volumes of dialysis buffer for 16 hours.

The dialyzed suspension was then centrifuged at 41,000 X g for 20 minutes. The supernatant was filtered through a 0.45 μ m filter and the conductivity was adjusted to less than 3.6 μ MHO with H₂O. The preparation was then loaded onto a Q-Sepharose FF column (Pharmacia) at a linear flow rate of 1.4 cm/min. All subsequent operations were at a flow rate of 20 cm/min. Protein elution was monitored continuously at following the optical density of the eluent at 280 nm. The column was then washed with buffer A (20 mM Tris, pH 8, 0.5 mM EDTA, 0.5 mM DTT, 10% glycerol) until optical density at 280 nm returned to baseline. Bound proteins were eluted by running a gradient against buffer B (1 M NaCl, 20 mM Tris, pH 8, 0.5 mM EDTA, 0.5 mM DTT, 10% glycerol). The gradient went to 25% buffer B over 50 minutes. Fractions were assayed for GMP synthetase activity and those with appreciable activity were pooled.

Ammonium sulfate was added slowly to the pooled fractions from the Q-Sepharose column (1.94 grams per 10 ml). The solution was then filtered through a 0.8 μ m filter. A Poros PE phenyl ether (PerSeptive Biosystems, Cambridge, MA) column was equilibrated against buffer C (20 mM Tris pH 8, 0.5 mM DTT, 10% glycerol, 19.4% ammonium sulfate) at a linear flow rate of 1.7 cm/min.

The protein was then loaded onto the column which was then washed with the equilibration buffer until optical density returned to baseline. This and subsequent operations were at 3.4 cm/min. A 0-60% buffer D (20 mM Tris pH 8, 0.5 mM DTT, 10% glycerol) gradient was run over a 36 minute interval. Fractions were assayed for GMP synthetase activity and those with appreciable activity were pooled. The protein was concentrated to approximately 2 mg/ml using a YM 30 membrane (Amicon) in a stir cell. The protein preparation was mixed with 1 part glycerol and frozen at -80C. The purified protein had kinetic and biochemical properties identical to those described of the purified human GMP synthetase from A3.01 cells described in Example 1. The specific activity of the enzyme was 4.4 μ mol AMP produced/min/mg.

The above procedure was repeated on several occasions and samples of recombinant human GMP synthetase with specific activities in the range of 3.0-4.4 μ moles AMP produced/min/mg were routinely obtained.

EXAMPLE 5

Assay for Inhibitors of Human GMP Synthetase

The source of enzyme was a baculoviral expressed human GMP synthetase obtained as described above. Alternatively, purified enzyme from A3.01 cells obtained as described in Example 1 was also used. Purified human GMP

synthetas enzyme was used in testing. The specific activity of the enzyme used was between 2.5 to 3.0 $\mu\text{mol AMP produced/min/mg}$. Two procedures were used for assaying enzyme activity in the presence of a suspected inhibitor.

5 Procedure #1: Spectrophotometric-coupled assay

 The formation of AMP was measured by coupling the GMP synthetase reaction to three auxiliary enzymes: these were AMP kinase, pyruvate kinase and lactate dehydrogenase. In addition to the substrates for GMP synthetase, also included in the assay mix were phosphoenol pyruvate (substrate for pyruvate kinase) and NADH (substrate for lactate dehydrogenase). Through the activities of these auxiliary enzymes, the formation of AMP was measured by the eventual oxidation of NADH which was monitored by the decrease of absorbance at 340 nm. This procedure could be automated in a high throughput screen. Because this assay is an indirect assay and an active compound can be inhibiting any or all four enzymes involved in the assay, it is used as a primary screen for quickly identifying potentially active inhibitors.

20 Procedure #2: Radioactive (direct) assay

 In this assay, radiolabeled XMP or ATP was used and the formation of radioactive GMP or AMP was measured directly after separation of substrates and products by thin-layer chromatography. This assay was used to confirm results from the primary screen. Typically, a suspected inhibitor was dissolved and diluted in DMSO and the final DMSO concentration in the assay was 10%.

Assay #1: Spectrophotometric-coupled assay for initial screen

Materials: Stock solutions are prepared and stored frozen until used.

	<u>Reagent</u>	<u>Concentration</u>
30	1. Tris.HCl, pH 7.8	750 mM
	2. MgCl_2	100 mM
	3. ATP	400 μM
	4. XMP	200 μM
35	5. glutamine	2000 μM
	6. phosphoenolpyruvate	5 mM
	7. NADH	1.5 mM
	8. KCl	80 mM
	9. dithiothreitol	1 M
40	10. compound or extract of suspected inhibitor	0 to 200 μM or 50 $\mu\text{g/ml}$ in DMSO (10% DMSO final)
	11. AMP kinase	1 mg/ml (2000 U/ml)
	12. pyruvate kinase	10 mg/ml (2000 U/ml)

-26-

13. lactate dehydrogenase 10 mg/ml (8500 U/ml)
 14. GMP synthetase 20 µg/ml

5 Method

1. Prepared assay mix by mixing 1 ml each of reagents 1-8, 0.5 ml of reagent 9 and 0.1375 ml of distilled H₂O. This is solution A.
 2. Pipetted 2.764 ml of solution A into a tube and warm to 40°C.
 3. Diluted 50 µl of compounds/extracts (reagent 10) into UV-quality disposable cuvettes.
 4. Prepared auxiliary enzyme mix by mixing 25 µl of each of reagents 11 and 12, and 12.5 µl of reagent 13.
 5. Added 20 µl of auxiliary enzyme mix (from step 4) to 2.764 ml of warm assay mix from step 2.
 6. Aliquoted 435 µl of the warm assay/auxiliary enzyme mix from step 5 into the individual cuvettes from step 3 containing the suspected inhibitory compounds or extracts.
 7. Started reaction by the addition of 15 µl GMP synthetase to the cuvettes from step 6.
 20 The final concentrations of all assay components are shown below.

		<u>Final concentration (in assay)</u>
	<u>Reagent</u>	
	1. Tris.HCl, pH 7.8	75 mM
25	2. MgCl ₂	10 mM
	3. ATP	40 µM
	4. XMP	20 µM
	5. glutamine	200 µM
	6. pyruvate	0.5 mM
30	7. NADH	0.15 mM
	8. KCl	8 mM
	9. dithiothreitol	50 mM
	10. compound or extract of suspected inhibitor	0 to 200 µM or 50 µg/ml in DMSO (10% DMSO final)
35	11. AMP kinase	2.5 µg/ml
	12. pyruvate kinase	25 µg/ml
	13. lactate dehydrogenase	1.25 µg/ml
	14. GMP synthetase	0.6 µg/ml

- 40 The assay was run at 40°C, 10 min, 0.5 ml final volume, monitoring continuous change in OD₃₄₀. A negative control was run using 50 µl of DMSO instead of the suspected inhibitory compound or extract.

-27-

Assay #2: Radioactive (direct) assay for confirmation screenMaterials:

	<u>Reagent</u>	<u>Concentration</u>
5	1. Tris.HCl, pH7.8	750 mM
	2. MgCl ₂	100 mM
	3. ATP	400 μ M
	4. XMP	2.5 mM
10	5. ¹⁴ C-XMP	1 mM (50 mCi/mmol)
	6. glutamine	2 mM
	7. dithiothreitol	1 M
	8. compound or extract of suspected inhibitor	0 to 200 μ M or 500 μ g/ml in DMSO
15	9. GMP synthetase	2.57 μ g/ml

Method

1. Prepared assay mix by mixing 60 μ l each of reagents 1, 2, 3, and 6, 30 μ l of reagent 7, 4.8 μ l of reagent 5, 2.9 μ l of reagent 4, and 122.3 μ l of distilled H₂O. This is solution A.
 2. Warmed assay mix (solution A) to 40°C.
 3. Diluted 3 μ l of compounds/extracts into 0.4 ml Eppendorf tubes.
 4. Aliquoted 20 μ l of warm assay mix from step 2 into the individual tubes containing the suspected inhibitors.
 5. Started the reaction by the addition of 7 μ l of GMP synthetase.
 6. Stopped the reaction after 10 minutes with 3 μ l 500 mM EDTA.
 7. Spiked reaction with 3 μ l of standards of GMP and XMP (125 mM each).
 8. Streaked 5 μ l assay mix (twice, total 10 μ l) onto PEI cellulose TLC plates (previously cut into 2.5 cm x 20 cm).
 9. Chromatographed in 2 M formic acid.
 10. Visualized GMP band by UV lamp.
 11. Excised GMP band and added 4 ml scintillation cocktail (Beckmann Ready-safe).
 12. Determined ¹⁴C-GMP formation by liquid scintillation counting.
- The assay was run at 40°C for 10 minutes in a final volume of 30 μ l. A negative control was run using 3 μ l of DMSO instead of the suspected inhibitory compound or extract. Several compounds which inhibited GMP synthetase by 50% or more under these conditions were identified using this screening procedure. The IC₅₀ of some of these compounds is shown below.

Compound	IC ₅₀
A	30 μ M
B	3 μ M
C	0.5 μ M
5 D	7 μ M
E	3 μ M
F	10 μ M

EXAMPLE 610 Generation of Anti-GMP Synthetase Antibody

An ~300 base pair *Hind* III/*Sac* I fragment from the middle of the coding region of clone 6 (GMPS.6) obtained in Example 2 (see Figure 4) was subcloned into the bacterial expression vector pGEX-2T (Pharmacia) and expressed as a glutathione transferase fusion protein in SURE bacteria. This fusion protein was isolated using a glutathione sepharose column according to Pharmacia instructions. A one liter culture yielded 1.5 mg of protein, which was used to immunize rabbits according to an approved protocol. Rabbit sera were collected and the immunoglobulin fraction was isolated. Purified human GMP synthetase was used in immunoblot analysis to test for the presence of antibodies against human GMP synthetase. In this fashion polyclonal rabbit antibodies were obtained.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity and understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Syntex (USA) Inc.
 - (A) ADDRESSEE: Syntex (USA) Inc.
 - (B) STREET: 3401 Hillview Avenue
 - (C) CITY: Palo Alto
 - (D) STATE: California
 - (E) COUNTRY: USA
 - (F) ZIP: 94304
 - (G) TELEPHONE: (415)-855-5160
 - (H) TELEFAX: (415)-496-3529
- (ii) TITLE OF INVENTION: Cloning and Expression of Human GMP Synthetase, its use in Screening for Inhibitors of Human GMP Synthetase and Inhibitors of Human GMP Synthetase
- (iii) NUMBER OF SEQUENCES: 11
- (iv) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (v) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 2237 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (iv) ORIGINAL SOURCE:
 - (G) CELL TYPE: Lymphoblast
 - (H) CELL LINE: A3.01
- (v) IMMEDIATE SOURCE:
 - (B) CLONE: GMPS.6

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GAATTCGGCA	CGAGCTCCTC	TCGGCTGCCG	GCTGCTCCTC	GACCAGSCCT	CCTTCTCAAC	60
CTCAGCCCGC	GGCGCCGAC	CTTCCGGCAC	CCTCCGCCCC	CGTCTCGTAC	TGTGCGCGTC	120
ACCGCCGCGG	CTCCGGCCCT	GGCCCGCATG	GCTCTGTGCA	ACGGAGACTC	CAAGCTGGAG	180
AATGCTGGAG	GAGACCTTAA	GGATGGCCAC	CACCACTATG	AAGGAGCTGT	TGTCATTCTG	240
GATGCTGGTG	CTCAGTACGG	GAAAGTCATA	GACCGAAGAG	TGAGGGAAC	GTTCTGTGAG	300
TCTGAAATTT	TCCCTTGGGA	AACCCAGCA	TTTGCTATAA	AGGAACAAGG	ATTCCGTGCT	360
ATTATCATCT	CTGAGGAGAC	TAATTCGTG	TATGCTGAAG	ATGCTCCCTG	GTTTGATCCA	420
GCAATATTCA	CTATTGGCAA	GCCTGTTCTT	GGAATTGTCT	ATGGTATGCA	GATGATGAAT	480
AAGGTATTTG	GAGGTACTGT	GCACAAAAAA	AGTGTGAGAG	AAGATGGAGT	TTTCAACATT	540
AGTGTGGATA	ATACATGTTT	ATTATTACAG	GGCCTTCAGA	AGGAAGAAGT	TGTTTTGCTT	600
ACACATGGAG	ATAGTGTAGA	CAAAAGTAGT	GATGGATTCA	AGGTTGTGGC	ACGTTCTGGA	660
AACATAGTAG	CAGGCATAGC	AAATGAATCT	AAAAAGTTAT	ATGGAGCACA	GTTCCACCTC	720
GAAATTTGCC	TTACAGAAAA	TGGAAAAATA	ATACTGAAGA	ATTTCCTTTA	TGATATAGCT	780
GGATCAGTGT	GAACCTTCAC	CGTGCAGAAC	AGAGAACCTG	AGTGTATTCT	AGAGATCAAA	840
GAGAGAGTAG	GCACGTCAAA	AGTTTTGGTT	TTACTCAGTG	GTGGAGTAGA	CTCAACAGTT	900
TGTACAGCTT	TGCTAAATCG	TGCTTTGAAC	CAAGAACAAG	TCATTGTGCT	GCACATTGAT	960
AATGGCTTTA	TGAGAAAAAG	AGAAAGCCAG	TCTGTTGAAG	AGGCCCTCAA	AAAGCTTGGG	1020
ATTCAAGTCA	AAGTGATAAA	TGCTGCTCAT	TCTTTCTACA	ATGGAACAAC	AACCTACCA	1080
ATATCAGATG	AAGATAGAAC	CCCACGGAAA	AGAATTAGCA	AAACGTTAAA	TATGACCACA	1140
AGTCCTGAAG	AGAAAAGAAA	AATCATTGGG	GATACTTTTG	TTAAGATTGC	CAATGAAGTA	1200
ATTGGAGAAA	TGAACCTTGAA	ACCAGAGGAG	GTTTTCTCTG	CCCAAGGTAC	TTTACGSCCT	1260
GATCTAATTG	AAAGTGCAATC	CCTTGTTGCA	AGTGGCAAGG	CTGAACCTAT	CAAAACCCAT	1320
CACATAGACA	CAGAGCTCAT	CAGAAAGTTG	AGAGAGGAGG	GAAAAGTAA	AGAACCTCTG	1380
AAAGATTTTT	ATAAAGATGA	AGTGAGAATT	TTGGGCGAGG	AACCTTGACT	TCCAGAAGAG	1440
TTAGTTTCCA	GGCATCCATT	TCCAGGTCCT	GGCCTGSCAA	TCAGAGTAAT	ATGTGCTGAA	1500
GAACCTTATA	TTTGTAAGGA	CTTTCTTGAA	ACCAACAATA	TTTTGAAAT	AGTAGCTGAT	1560
TTTTCTGCAA	GTTGTTAAAA	GCCACATACC	CTATTACAGA	GAGTCAAAAG	CTGCACAACA	1620
GAAGAGGATC	AGGAGAAGCT	GATGCAAATT	ACCACTCTGC	ATTCAGTGAA	TGSCCTTCTG	1680
CTGCCAATTA	AAACTGTAGG	TGTGCAGGGT	GACTGTGCTT	CCTACAGTTA	CGTGTGTGGA	1740
ATCTCCAATA	AAGATGAACC	TGACTGGGAA	TCATTATATT	TTCTGGCTAG	GCTTATACCT	1800
CGCATGTGTC	ACAACGTTAA	CAGAGTTGTT	TATATATTTG	GCCCACCACT	TAAAGAACTT	1860
CCTACAGATG	TTACTCCAC	TTTCTTGACA	ACAGGGGTGC	TCAGTACTTT	ACSCCAAGCT	1920
GATTTTGAGG	CCCATTAACAT	TCTCAGGGAG	TCTGGGTATG	CTGGGAAAA	CAGCCAGATG	1980

COGGTGATTG TGACACCATT ACATTTTGAT CGGGACCCAC TTCAAAAGCA GCCTTCATGC 2040
CAGAGATCTG TGGTTATTCG AACCTTTATT ACTAGTGACT TCATGACTGG TATACCTGCA 2100
ACACCTGGCA ATGAGATCCC TGTAGAGGTG GTATTAAAGA TGGTCACTGA GATTAAGAAG 2160
ATTCTCGGTA TTTCGCGAAT TATGTATGAC TTAACATCAA AGCCCCCAGG AACTACTGAG 2220
TGGGAGTAAT AACTTC 2237

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 11 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: peptide

- (iii) HYPOTHETICAL: NO

- (v) FRAGMENT TYPE: internal

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Asn Ile Val Ala Gly Ile Ala Asn Glu Ser Lys
1 5 10

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 17 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: peptide

- (iii) HYPOTHETICAL: NO

- (v) FRAGMENT TYPE: internal

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Ala Leu Asn Gln Glu Gln Val Ile Ala Val Xaa Ile Asp Asn Gly Phe
1 5 10 15
Met

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: peptide

- (iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Val Ile Asn Ala Ala His Ser Phe Tyr Asn Gly Thr Xaa Xaa Leu Pro
1 5 10 15
Ile Ser Asp Glu Asp
20

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Ile Ile Gly Asp Thr Phe Val
1 5

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Val Ile Glu Pro Leu Lys Asp Phe Ile Lys Asp Glu Val Arg
1 5 10

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Val Ile Glu Pro Leu Lys Asp Phe Ile Lys Asp Glu Val Arg
1 5 10

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Pro Phe Pro Gly Pro Gly Leu Ala Ile
1 5

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Phe Ile Thr Ser Asp Phe Met Thr Gly Ile Pro Ala Thr Pro Gly Asn
1 5 10 15
Glu Ile Xaa Val
20

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Ile Met Tyr Asp Leu Thr Ser Lys Pro Pro Gly Thr Thr Glu
 1 5 10

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 693 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Met Ala Leu Cys Asn Gly Asp Ser Lys Leu Glu Asn Ala Gly Gly Asp
 1 5 10 15
 Leu Lys Asp Gly His His His Tyr Glu Gly Ala Val Val Ile Leu Asp
 20 25 30
 Ala Gly Ala Gln Tyr Gly Lys Val Ile Asp Arg Arg Val Arg Glu Leu
 35 40 45
 Phe Val Gln Ser Glu Ile Phe Pro Leu Glu Thr Pro Ala Phe Ala Ile
 50 55 60
 Lys Glu Gln Gly Phe Arg Ala Ile Ile Ile Ser Gly Gly Pro Asn Ser
 65 70 75 80
 Val Tyr Ala Glu Asp Ala Pro Trp Phe Asp Pro Ala Ile Phe Thr Ile
 85 90 95
 Gly Lys Pro Val Leu Gly Ile Cys Tyr Gly Met Gln Met Met Asn Lys
 100 105 110
 Val Phe Gly Gly Thr Val His Lys Lys Ser Val Arg Glu Asp Gly Val
 115 120 125
 Phe Asn Ile Ser Val Asp Asn Thr Cys Ser Leu Phe Arg Gly Leu Gln
 130 135 140
 Lys Glu Glu Val Val Leu Leu Thr His Gly Asp Ser Val Asp Lys Val
 145 150 155 160
 Ala Asp Gly Phe Lys Val Val Ala Arg Ser Gly Asn Ile Val Ala Gly
 165 170 175
 Ile Ala Asn Glu Ser Lys Lys Leu Tyr Gly Ala Gln Phe His Pro Glu
 180 185 190
 Val Gly Leu Thr Glu Asn Gly Lys Val Ile Leu Lys Asn Phe Leu Tyr
 195 200 205
 Asp Ile Ala Gly Cys Ser Gly Thr Phe Thr Val Gln Asn Arg Glu Leu
 210 215 220
 Glu Cys Ile Arg Glu Ile Lys Glu Arg Val Gly Thr Ser Lys Val Leu
 225 230 235 240

Val Leu Leu Ser Gly Gly Val Asp Ser Thr Val Cys Thr Ala Leu Leu
 245 250 255
 Asn Arg Ala Leu Asn Gln Glu Gln Val Ile Ala Val His Ile Asp Asn
 260 265 270
 Gly Phe Met Arg Lys Arg Glu Ser Gln Ser Val Glu Glu Ala Leu Lys
 275 280 285
 Lys Leu Gly Ile Gln Val Lys Val Ile Asn Ala Ala His Ser Phe Tyr
 290 295 300
 Asn Gly Thr Thr Thr Leu Pro Ile Ser Asp Glu Asp Arg Thr Pro Arg
 305 310 315 320
 Lys Arg Ile Ser Lys Thr Leu Asn Met Thr Thr Ser Pro Glu Glu Lys
 325 330 335
 Arg Lys Ile Ile Gly Asp Thr Phe Val Lys Ile Ala Asn Glu Val Ile
 340 345 350
 Gly Glu Met Asn Leu Lys Pro Glu Glu Val Phe Leu Ala Gln Gly Thr
 355 360 365
 Leu Arg Pro Asp Leu Ile Glu Ser Ala Ser Leu Val Ala Ser Gly Lys
 370 375 380
 Ala Glu Leu Ile Lys Thr His His Asn Asp Thr Glu Leu Ile Arg Lys
 385 390 395 400
 Leu Arg Glu Glu Gly Lys Val Ile Glu Pro Leu Lys Asp Phe His Lys
 405 410 415
 Asp Glu Val Arg Ile Leu Gly Arg Glu Leu Gly Leu Pro Glu Glu Leu
 420 425 430
 Val Ser Arg His Pro Phe Pro Gly Pro Gly Leu Ala Ile Arg Val Ile
 435 440 445
 Cys Ala Glu Glu Pro Tyr Ile Cys Lys Asp Phe Pro Glu Thr Asn Asn
 450 455 460
 Ile Leu Lys Ile Val Ala Asp Phe Ser Ala Ser Val Lys Lys Pro His
 465 470 475 480
 Thr Leu Leu Gln Arg Val Lys Ala Cys Thr Thr Glu Glu Asp Gln Glu
 485 490 495
 Lys Leu Met Gln Ile Thr Ser Leu His Ser Leu Asn Ala Phe Leu Leu
 500 505 510
 Pro Ile Lys Thr Val Gly Val Gln Gly Asp Cys Arg Ser Tyr Ser Tyr
 515 520 525
 Val Cys Gly Ile Ser Ser Lys Asp Glu Pro Asp Trp Glu Ser Leu Ile
 530 535 540
 Phe Leu Ala Arg Leu Ile Pro Arg Met Cys His Asn Val Asn Arg Val
 545 550 555 560
 Val Tyr Ile Phe Gly Pro Pro Val Lys Glu Pro Pro Thr Asp Val Thr
 565 570 575
 Pro Thr Phe Leu Thr Thr Gly Val Leu Ser Thr Leu Arg Gln Ala Asp
 580 585 590

Phe Glu Ala His Asn Ile Leu Arg Glu Ser Gly Tyr Ala Gly Lys Ile
595 600 605

Ser Gln Met Pro Val Ile Leu Thr Pro Leu His Phe Asp Arg Asp Pro
610 615 620

Leu Gln Lys Gln Pro Ser Cys Gln Arg Ser Val Val Ile Arg Thr Phe
625 630 635 640

Ile Thr Ser Asp Phe Met Thr Gly Ile Pro Ala Thr Pro Gly Asn Glu
645 650 655

Ile Pro Val Glu Val Val Leu Lys Met Val Thr Glu Ile Lys Lys Ile
660 665 670

Pro Gly Ile Ser Arg Ile Met Tyr Asp Leu Thr Ser Lys Pro Pro Gly
675 680 685

Thr Thr Glu Trp Glu
690

What is claimed is:

1. A purified human GMP synthetase.
2. A recombinant human GMP synthetase.
3. A recombinant polynucleotide comprising a nucleic acid sequence
5 derived from human GMP cDNA.
4. A polynucleotide comprising the sequence of GMPs.6.
5. A polynucleotide comprising the sequence of pcr.2S8A.
6. A polynucleotide probe comprising the polynucleotide of Claim 3
bound to a reporter molecule.
- 10 7. A polynucleotide probe comprising the polynucleotide of Claim 5
bound to a reporter molecule.
8. A recombinant vector comprising the polynucleotide of Claim 3.
9. A recombinant vector comprising the polynucleotide of Claim 4.
10. A host cell transformed with the vector of Claim 8.
- 15 11. A host cell transformed with the vector of Claim 9.
12. A cDNA library comprising the host cells of Claim 10.
13. A cDNA library comprising the host cells of Claim 11.
14. A DNA sequence encoding for human GMP synthetase.
15. A cDNA sequence encoding for human GMP synthetase.
- 20 16. Expression vectors comprising the DNA of Claim 14.
17. Expression vectors comprising the cDNA of Claim 15.
18. Host cells transformed with the expression vectors of Claim 16.
19. Host cells transformed with the expression vectors of Claim 17.
- 25 20. A method for producing human GMP synthetase comprising
incubating host cells transformed with an expression vector
containing a DNA sequence coding for human GMP synthetase under
conditions which allow expression of human GMP synthetase.
- 30 21. A method for producing human GMP synthetase comprising
incubating the host cells of Claim 18 under conditions which
allow expression of human GMP synthetase.
22. A method for producing human GMP synthetase comprising
incubating the host cells of Claim 19 under conditions which
allow expression of human GMP synthetase.
- 35 23. A method for purifying human GMP synthetase produced by host
cells comprising,
(a) homogenizing said host cells and isolating the cytosolic
component,
(b) fractionating said cytosolic component to its protein fraction
and
40 (c) subjecting said protein fraction to chromatographic
purification.
24. The method Claim 23 where said host cells are mammalian cells.
25. An antibody to human GMP synthetase.
- 45 26. A monoclonal antibody to human GMP synthetase.

27. An assay for inhibitors of human GMP synthetase comprising contacting a compound suspected of being said inhibitor with purified human GMP synthetase and measuring the activity of the human GMP synthetase.
- 5 28. An inhibitor of human GMP synthetase activity, said inhibitor having an IC_{50} of $5\mu M$ or less.
29. An inhibitor of human GMP synthetase, said inhibitor having an IC_{50} of $1\mu M$ or less.
- 10 30. A method of inhibiting human GMP synthetase activity comprising administering an effective amount of a compound having an IC_{50} of $5\mu M$ or less.
31. An inhibitor of human GMP synthetase activity identified by the process of contacting said inhibitor with purified human GMP synthetase and measuring the activity of said GMP synthetase.
- 15 32. The inhibitor of Claim 30 having an IC_{50} of less than $20\mu M$.
33. The inhibitor of Claim 30 having an IC_{50} of less than $10\mu M$.
34. The inhibitor of Claim 30 having an IC_{50} of $5\mu M$ or less.
35. The inhibitor of Claim 30 having an IC_{50} of $1\mu M$ or less.
- 20

Fig. 1-B

781	<u>AAC CAA GAA CAA CTC ATT GCT GAT</u>	CAC	ATT	GAT	ATG	AGC	AAA	CGA	GAA	AGC
782	<u>N Q E Q V I A V H I P N G P H R K R E S</u>									
783	<u>CAG TCT GTT GAA GCC CTA AAT GAT CTC AAT GAT GTC ATA AAT GCT GCT</u>									
784	<u>Q S V E E A L K K L G I Q V K V I N A A</u>									
785	<u>CAT TCT TTC TAC AAT GGA ACA ACC CTA CCA ATA TCA GAT GAA GAT AGA ACC GCA CGG</u>									
786	<u>H E F P H G T T A P A F A H D E R P R</u>									
787	<u>AAA AGA ATT AGC AAA ACC TTA AAT ATG ACC ACA AGT CCT GAA GAG AAA AGA ATC ATT</u>									
788	<u>K R I S K T L M M T T S P E E K R K I L</u>									
789	<u>GGG GAT ACT TTT GTT ATG ATT GGC AAT GAA GTA ATT GGA ATG AAC TTG AAA CCA GAG</u>									
790	<u>G D T F V K I A M E V I G E M L K P E</u>									
791	<u>GAG GTT TTC CTT GCC CAA GGT ACT TTA CGG CCT GAT CTA ATT GAA AGT GCA TCC CTT GTT</u>									
792	<u>E V F L A G G L E P D L I E S A B L V</u>									
793	<u>ACA AGT GGC AAA GCT GAA CTC ATC AAA ACC CAT CAC AAT GAC ACA GAG CTC ATC AGA AAG</u>									
794	<u>A S G K A E L I K T E M E M D T E L I R K</u>									
795	<u>TTG AGA GAG GAG GGA AAA GTA ATA GAA CCT CTG AAA GAT TTT CAT AAA GAT GAA GTG AGA</u>									
796	<u>L R E E G K V I E P L K D P H K E D E V R</u>									
797	<u>ATT TTG GGC AGA GAA CTT GCA CTT CCA GAA GAT TTA GTT TCC AGG CAT CCA TTT CCA GGT</u>									
798	<u>I L G R E L G L P E L V S R E P P P G</u>									
799	<u>CCT GGC CTG CCA ATC AGA GTA ATA TGT GCT GAA GAA CCA TAT ATT TGT AAG GAC TTT CCT</u>									
800	<u>P G L A I R V I C A E E P Y I C K D P P</u>									
801	<u>GAA ACC AAC AAT ATT TTG AAA ATA GTA GCT GAT TTT TCT GCA AGT GTT AAA AAG CCA CAT</u>									
802	<u>E T N M I L K I V A D F S A S V K K P E</u>									
803	<u>ACC CTA TTA CAG AGA GTC AAA GCC TGC ACA ACA GAA GAT CAG GAG AAG CTG ATG CAA</u>									
804	<u>T L L G R V K A C T E E D Q E K L M Q</u>									
805	<u>ATT ACC AGT CTG CAT TCA CTG AAT GGC TTC TTG CTG CCA ATT AAA ACT GTA GGT GTG CAG</u>									
806	<u>I T L S L M A L P I K T V G V Q</u>									
807	<u>GCT GAC TGT GGT TCC TAC AGT TAC GTG TGT GGA ATC TCC AGT AAA GAT GAT GAC TGG</u>									
808	<u>G D C R S Y S V C I S S K D E P D M</u>									

1621 GAA TCA CTT ATT TTT CTG GCT AGG CTT ATA CCT CCG ATG TGT CAC AAC GTT AAC AGA GTT
 541 E S L I P L A R L I P R H C H N V N R V
 1681 GTT TAT ATA TTT GGC CCA CCA GTT AAA GAA CCT CCT ACA GAT GTT ACT CCC ACT TTC TTG
 561 V Y I F G P P V K E P P T D V T P T F L
 1741 ACA ACA GGG GTG CTC AGT ACT TTA CGC CAA GCT TTT GAG GCC CAT AAC ATT CTC AGG
 581 T T G V L S T L R Q A D F E A H N I L R
 1801 GAG TCT GGG TAT GCT GGG AAA ATC AGC CAG ATG CCG GTG ATT TTG ACA CCA TTA CAT TTT
 601 E S G Y A G K I S Q M P V I L T P L H P
 1861 GAT CGG GAC CCA CTT CAA AAG CAG CCT TCA TGC CAG AGA TCT GTG GTT ATT CGA ACC TTT
 621 D R D P L Q K Q P S C Q R S V V I R T E
 1921 ATT ACT AGT EAC TTC ATG ACT GGT ATA CCT GGA ACA CCT GGC AAT GAG ATC CCT GTA GAG
 641 I T S D P F M T G I P A T P G N E I P Y E
 1981 GTG GTA TTA AAG ATG GTC ACT GAG ATT AAG AAG ATT CCT GGT ATT TCT CGA ATT ATG TAT
 661 V V L K M V T E I K K I P G I S R I M Y
 2041 GAC TTA ACA TCA AAG CCC CCA GGA ACT ACT GAG TGG GAG TATTAACCTTC
 681 D L T S R P P Q T T E E
 (8)
 (9)

Fig. 2

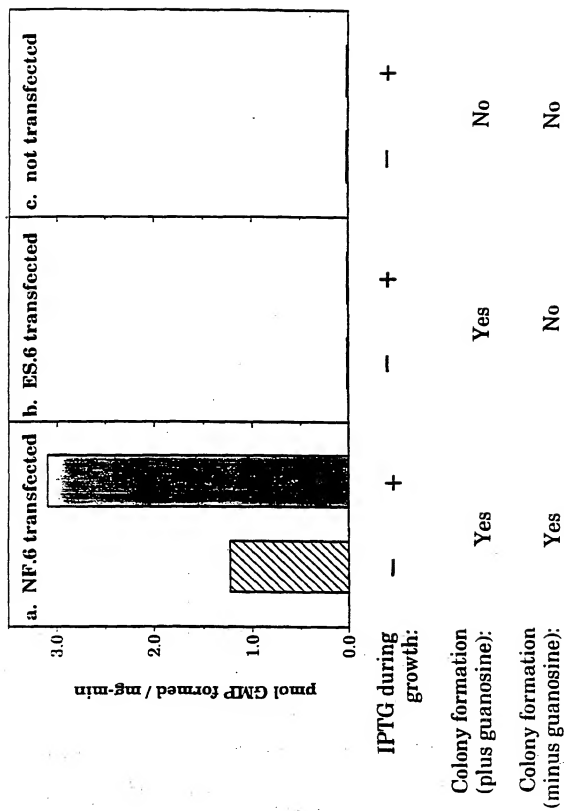
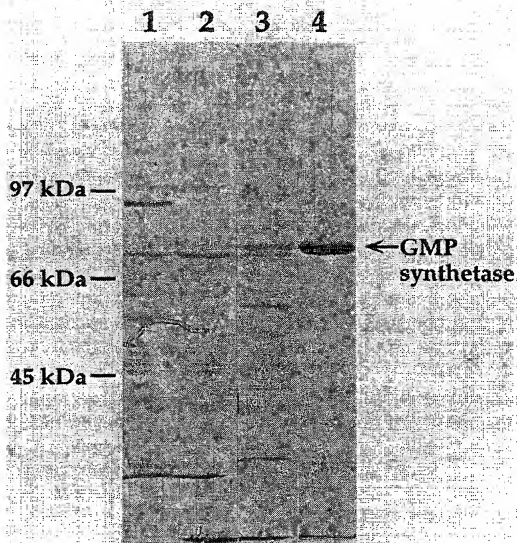
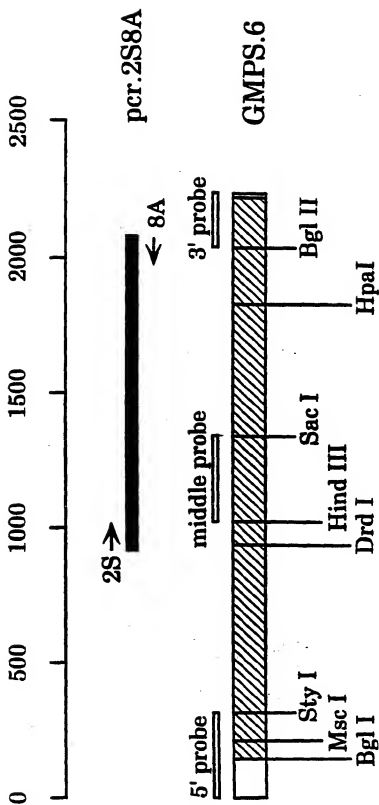


Fig. 3



6/6

Fig. 4



INTERNATIONAL SEARCH REPORT

 Inter Application No
 PC1703 95/03934

A. CLASSIFICATION OF SUBJECT MATTER		
IPC 6	C12N15/52 C12N5/10	C12N9/00 C07K16/40 G01N33/50 C12N1/21
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols)		
IPC 6 C12N C07K G01N		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	THE INTERNATIONAL JOURNAL OF BIOCHEMISTRY, vol.16, no.1, 1984 pages 117 - 120 PAGE T. ET AL. 'Human GMP synthetase' cited in the application see abstract see page 119, left column; table 2 ---	1,2, 27-35
X	J. BIOCHEM. (TOKYO), vol.102, no.4, October 1987 pages 893 - 902 HIRAI K. ET AL. 'Purification and characterization of GMP synthetase from Yoshida Sarcoma Ascites cells' cited in the application see page 898, right column see page 900, left column ---	1,2, 27-35
-/-		
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input type="checkbox"/> Patent family members are listed in annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "A" document member of the same patent family		
Date of the actual completion of the international search		Date of mailing of the international search report
24 July 1995		10.08.95
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patendaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016		Authorized officer Esen, J

Form PCT/ISA/218 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Intern at Application No

PCT/US 95/03934

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF BIOLOGICAL CHEMISTRY, vol.250, no.18, 1975, BALTIMORE, MD US pages 7372 - 7376 SPECTOR T. 'Studies with GMP synthetase from Ehrlich ascites cells' see page 7374, left column ---	28-35
Y	JOURNAL OF BACTERIOLOGY, vol.174, no.6, March 1992 pages 1883 - 1890 MANTSALA P. ET AL. 'Cloning and sequence of Bacillus subtilis purA and guaA, involved in the conversion of IMP to AMP and GMP' cited in the application see the whole document ---	1-35
Y	JOURNAL OF BIOLOGICAL CHEMISTRY, vol.266, no.25, October 1991, BALTIMORE, MD US pages 16448 - 16452 VAN LOOKEREN CAMPAGNE M. M. ET AL. 'Functional cloning of a Dictyostelium discoideum cDNA encoding GMP synthetase' cited in the application see the whole document ---	1-35
Y	JOURNAL OF BIOLOGICAL CHEMISTRY, vol.260, 1985 pages 8676 - 8679 TIEDEMAN A. A. ET AL. 'Nucleotide sequence of the guaA gene encoding GMP synthetase of Escherichia coli K12' cited in the application see the whole document ---	1-35
P,X	JOURNAL OF BIOLOGICAL CHEMISTRY, vol.269, no.38, 23 September 1994, BALTIMORE, MD US pages 23830 - 23837 HIRST M. ET AL. 'Human GMP synthetase' see the whole document ---	1-35
P,X	JOURNAL OF BIOLOGICAL CHEMISTRY, vol.270, no.13, 31 March 1995, BALTIMORE, MD US pages 7347 - 7353 NAKAMURA J. ET AL. 'Biochemical characterization of human GMP synthetase' see the whole document -----	1-35

3

Form PCT/ISA/219 (continuation of second sheet) (July 1993)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US/03934

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 30
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim 30 is directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

Fig. 1-B

281 GAC GAA GAA GAA GTC ATT GCT GAG CAC ATT GAT AAT GGC TTT ATG AGA AAA CGA GAA AGC
 282 M D E Q V I A V E J D H G F M N R S P
 283 CAC TCT GTT GAC GAG CCC CTC AAA AAG CTT CAG CTC AAA GCG ATA AAT GCT GCT
 284 Q Y E R H A L K K L G I Q V K Y I H A A
 285 CAT TCT TTC TAC AAT GAA ACA ACA ACC CTA CCA ATA TCA GAT GAA GAT AGA ACC CCA TCG
 286 H S X Y M H X T L P I S D X A A
 287 AAA AGA ATT AGC AAA AGC TTA AAT ATG ACC ACA AAT GCT GAA GAG AAA ACA AAA AGC ATT
 288 K R I S K T L E M T T S P E E K R K I
 289 GGG GAT ACT TTT GTT AAG ATT GGC AAT GAA GTA ATT GGA GGA AAG AAC TTT AAA CCA CAG
 290 G D Y Y Y X I A M N Y I G K L I K
 291 GAG GTT TTC CTT GGC GAA GGT ACT TTA CGE GCT GAT CTA ATT GAA AGT GGA TCC CTT GAT
 292 E V P D A G G T L E P D I E A A G L Y
 293 GCA AAT GGC AAA GCT GAA CTC ATC AAA ACC CAG CAC AAT GAC ACA GAG CTA ATC AGA AAG
 294 A S Q X A E L I X T E M M D W S L I E K
 295 TTG AGA GAG GAG GGA AAA GTA ATA GAA GCT CTG AAA GAT TTT CAT AAA GAT GAA GTG AGA
 296 L R E E G K Y I E L X D P H K D X Y E
 297 ATT TTG GAC AGA GAA CTT GCA CTT CCA GAA GAG TTA GTC TCT AAG CAT CTA TTT CCA GGT
 298 I L G R X L G L P E E L V E E E P L E
 299 CTT GGC CTG GCA ATC AGA GTA AAT TGT GCT GAA GAA CTT TAT ATT TGT AAG GAC TTT CTT
 300 E Q A A A I R V I C A E X X P Y I C X D P
 301 GAA ACC AAC AAT ATT TTG AAG AAA ATA GTA GAT TTT GCT GCA AGT GTT AAA AAG CCA CAT
 302 E T E M I L E I V A D P E A G V K E P H
 303 ACC CTA TTA CAG ACA GCA GGC TGC ACA GAA GAG GAT CAG GAG CAG CTA GAG CAA
 304 T L L Q K V K A C T E E E D G E K L M D
 305 ATT ACC AGT CTG CAT TCA CTG AAT GGC TTC CTG CCA ATT AAA ACT GTA GAT GTG CAG
 306 I Y E L M A L L P I K Y G V Q Y Q
 307 GGT GAT TTT TCT TAC AGT TAC GAT ATC TCC AGT AAA GAT GAA CTT GAC TGG
 308 G D C E H T Y Y V C G I S B K D E P D W

1621 GAA TCA CTT ATT TTT CTG GCT AGG CTT ATA CTT CAC ATG TGT CAC AAC GTT AAC ACA GTT
 541 E S L I P L A R L I P A M C M M V M R V
 1641 GTT TAT ATA TTT GGC CCA CCA GGT AAA GAA CCA CTT CCA GAT GAT GAT GAT GAT GAT GAT GAT GAT
 541 V Y I P G P P V K E P T D V T P T P L
 1741 ACA ACA GGG GTG CTT AGT ACT TTA CCG CAA GCT GAT TTT GAG GGC CAT AAC ATT CTC AGG
 541 T T G V L S T L R Q A D P E A E M I L E
 1801 GAG TCT GGG TAT GCT GGG AAA ATC AGC CAG ANG CCG GTG ATT TTS ACA CCA TTA CAT TTT
 601 E S G Y A G K I G Q M P V I L T P L H P
 1861 GAT CCG GAC CCA CTT CAA AAG CAG CCT TCA TCC CAG AGA TCT GTG GAT ATT CCA ACC TTT
 621 D R D P L Q K Q P S C Q R S V V I M T X
 1921 ATT ACT AGT TAC TTT AAG ACT GAT ATA CTT GGA ACA OCT GGC AAT GAG ATC CTT GTA GAG
 541 I T S R T M T G I P A T P G M E I P V E
 1981 GTG GTA TTA AAG ANG GTC ACT GAG ATT AAG AAG ATT CTT GAT ATT TCT CGA ATT ATG TAT
 641 V V L X M V T E I K E I P G I S R I M Y
 2011 GAC TTA ACA TCA AAG CCG CCA GGA ACT ACT GAG TGG GAG TAAATACCTC
 661 H I T E K P P S T N E

Fig. 2

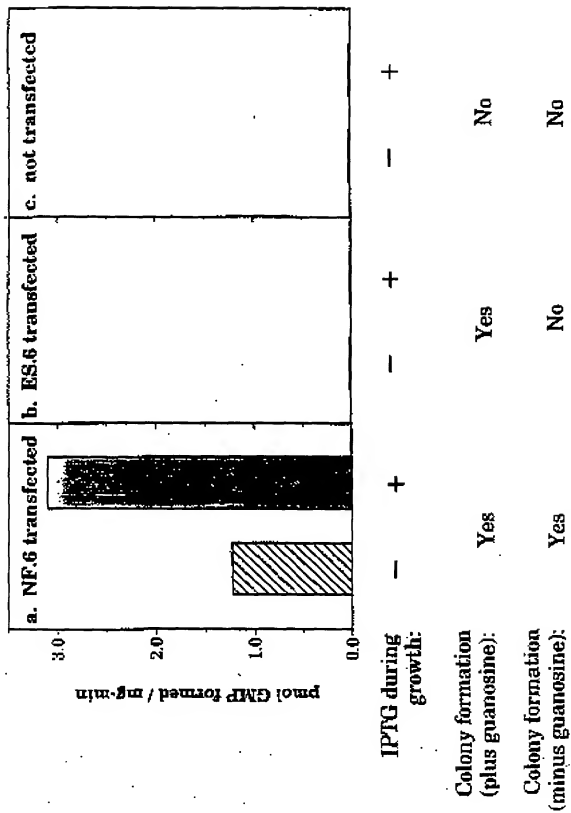
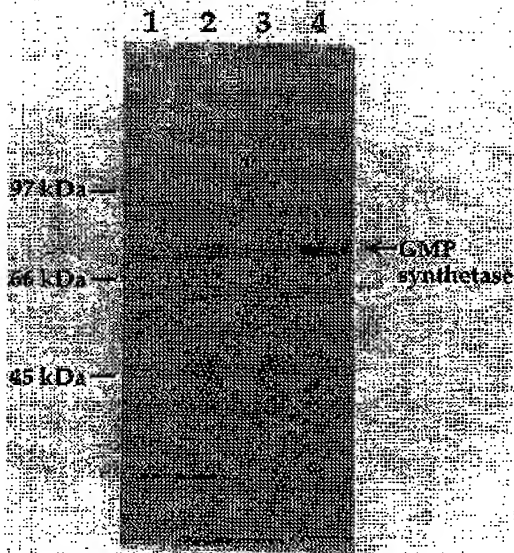


Fig. 3



6/6

Fig. 4

